

DNA vaccines for allergy treatment

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Abstract

In the past 10 years, a great number of studies have demonstrated that injection of plasmid DNA coding for certain genes results in the induction of humoral and cellular immune responses against the respective gene product. This vaccination approach covers a broad range of possible applications, including the induction of protective immunity against viral, bacterial, and parasitic infections, and it opens new perspectives for treatment of cancer. Surprisingly, DNA immunization also turned out as a promising novel type of immunotherapy against allergy. In this paper, we describe the construction of DNA vaccines for application in allergy models. Beyond, we offer a palette of recently developed modulations to optimize DNA vaccines for allergy treatment by increasing their immunogenicity and minimizing their anaphylactic potential.

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1. Introduction

The first publications demonstrating that injection of plasmid DNA is able to induce potent humoral and cellular immune responses against the encoded genes [1,2] opened a new era of vaccine research. In vivo transfection of epidermal tissue with the gene gun obviously resulted in transcription and translation of genes followed by proper presentation of gene products to the immune system. Considering the similarity of these events to the endogenous pathways involved in anti-viral responses, it was originally postulated that this new method would primarily be suitable for vaccination against viral infections. However, since the first publications, a large number of reports have demonstrated the enormously broad range of possible applications. Genetic immunization proved to induce protective immunity against bacterial and parasitic infections [3,4] and also offered new perspectives for the treatment of cancer [5] and allergic diseases [6,7].

Although the precise mechanisms involved in the induction of an immune response to antigens expressed by host cells following DNA immunization have yet to be determined, we have a fairly good understanding of antigen processing, presentation, and recognition by cells of the immune system.

In general, exogenous antigens are presented in the context of major histocompatibility complex (MHC) class II molecules. Internalization of proteins from the extracellular space is followed by denaturation and degradation resulting in peptide fragments. A small fraction of suitable, 12–20 amino acid long peptides, will bind to MHC class II molecules, which are transported to the cell surface and are recognized by antigen-specific CD⁴⁺ T-cells. This typical pathway for antigens from the extracellular milieu is apparently not connected with the processing of endogenous proteins.

In contrast, CD⁸⁺ lymphocytes recognize a complex of the MHC class I molecule with an embedded 8–12 amino acid long peptide antigen. Peptides presented on MHC class I molecules are usually generated by proteasomal processing of intracellular proteins. These peptides are delivered into the endoplasmic reticulum (ER) via the transporter associated with antigen presentation

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(TAP), bound to MHC class I molecules, and delivered to the cell surface.

The downstream events involved in the activation of T-cells require the presentation of antigen by professional antigen presenting cells (APCs) in the context of certain co-stimulatory signals. The absence of appropriate co-stimuli results in anergy or activation-induced cell death by apoptosis [8]. A number of studies have demonstrated that of all cell types, dendritic cells (DCs) are most effective in recognizing and responding to various “danger” signals [9–11]. In general, such signals result from tissue and/or cell damage, but also molecules of bacterial origin such as lipopolysaccharide (LPS) and even bacterial DNA itself can act as “danger” signals. These DNA sequences, also designated as CpG motifs, contain unmethylated CpG dinucleotides flanked by two 5' purines and two 3' pyrimidines and were shown to stimulate the innate immune system to produce type-1 helper T lymphocyte (Th1)-promoting cytokines [12,13].

However, this widely accepted picture of immune mechanisms still does not completely solve the question how DNA vaccines manage to trigger different branches of the immune system. Initial studies, which indicated long-term gene expression and translation of proteins by transfected myocytes, led to the conclusion that these cells had been recruited for antigen presentation [14]. Cytotoxic responses were thought to result from production, processing, and presentation of plasmid-derived protein, whereas release (or secretion) of soluble antigen provided material for T helper and B-cell activation as a prerequisite for specific antibody production. However, as myocytes lack crucial co-stimulatory molecules, presentation of antigens solely by muscle cells should result in immunological ignorance or tolerance. Several publications addressing the induction of cytotoxic T lymphocyte (CTL) responses in bone marrow chimeras following DNA immunization presented a different picture: Intramuscular (i.m.) as well as intradermal (i.d.) injections of plasmid DNA revealed that initiation of cell-mediated immune responses requires antigen presentation by professional APCs [15–19]. Acquisition of antigens by APCs is not fully understood, however, the mechanism of cross-priming [20–23] offers an attractive explanation of how soluble antigens (e.g., translated by muscle cells or keratinocytes) gain access to the MHC class I restricted pathway.

1.1. DNA immunization offers unique possibilities for vaccine development

One of the major advantages of DNA immunization is undoubtedly the possibility to co-deliver information together with the antigen-coding sequence on the plasmid DNA. Recently, several strategies have successfully been used to modulate the immune response after DNA immunization, such as: (i) modification of the vector by

inserting or deleting various regulatory sequences, (ii) co-delivery of genes or adjuvant molecules with regulatory and/or stimulatory properties, and (iii) different methods and sites of gene delivery.

These possibilities, either as single approaches or in combinations, opened up new perspectives for both vaccine development as well as basic immunological research [3–5].

1.2. Allergy and DNA immunization

The prevalence for IgE-mediated allergic diseases appears intimately linked to a dysregulation in the balance of Th1 and Th2 lymphocytes [24–26]. Current immunotherapeutic approaches for the treatment of allergic diseases generally aim at either inducing tolerance, or redirecting the immune response towards a Th1-type reaction.

Regarding the latter, DNA immunization has yielded promising results as a novel type of immunotherapy. I.m. or i.d. injection of plasmid DNA has repeatedly been shown to induce a Th1-biased immune response with promotion of IFN- γ -producing CD4⁺ T-cells, elevated production of IgG2a, and inhibition of IgE [6,7,27,28]. In animal studies, these protocols induced protective and therapeutic effects with model antigens as well as clinically relevant allergens [6,7,29–32].

Since cDNAs of many important allergens covering the epitope complexity of most relevant allergen sources are already on hand, the prerequisites for construction and clinical evaluation of DNA-based-specific allergy vaccines are available [33]. However, before DNA-based allergy vaccination can be taken into clinical application, two concerns need to be addressed: (i) the danger of autoimmune responses caused by excessive Th1 immune activation induced by DNA vaccination may be overcome by the development of strictly allergen-specific vaccination protocols. (ii) Anaphylactic side effects known from specific immunotherapy (SIT), caused by pre-existing and/or newly “therapy-induced” IgE antibodies, should be avoided during the course of the therapy.

The following sections offer effective strategies to solve these concerns and additionally introduce a panel of modulations for the development of novel types of DNA vaccines.

1.3. Vector modification, immunomodulation, and co-stimulation

The anti-allergic effect of DNA vaccines depends on recruitment of Th1 cells and strong immunogenicity is a necessary prerequisite for DNA-based desensitization approaches. Therefore, the expression level, the form of the antigen, and the immunostimulatory “danger” signals are of crucial importance.

The expression level is mainly influenced by features of the vector itself. Moreover, suboptimal codon usage of transfected cells concerning heterologous genes (which is the case with DNA vaccines encoding plant allergens and mammal cells as the target of the vaccine) can severely diminish protein expression. Therefore, target antigen sequences should routinely be recoded for DNA vaccines used for allergy treatment.

Considering that antigen presentation is crucial for immunogenicity, the form of the translated antigen plays an important role. Targeting proteins to the ER using a leader sequence results in enhanced secretion and increased amounts of antigen available for MHC class II presentation.

To increase the safety of DNA vaccines, production of native determinants of the allergen must be avoided to prevent anaphylactic responses induced by cross-linking of pre-existing or vaccine-induced IgE antibodies on mast cells.

However, to guarantee the recruitment of allergen-specific Th1 cells, T-cell epitopes must not be destroyed. Three approaches fulfill these requirements: (i) cutting the allergen gene into overlapping fragments, which lack any antigenic determinant of the native allergen but display the original repertoire of T-cell epitopes, (ii) fusing the allergen with ubiquitin thus fragmenting the antigen and destroying its native structure, and (iii) creating CpG enriched vectors, inserting additional CpG motifs by recoding the gene sequence (“CpGizing”), or co-injecting CpG-oligodeoxynucleotides (CpG-ODN) together with the DNA vaccine, which leads to reduced antibody responses and increased Th1-biased cellular reactions.

2. Description of methods

2.1. Generation and preparation of DNA vaccines

2.1.1. Construction of a versatile vector

Vectors for DNA immunization should enable high expression levels of the target protein in mammalian

cells but must not replicate or integrate into the host chromosome. Many commercially available mammalian expression vectors fulfill these requirements. This instruction focuses on the high copy number plasmid pCI (Promega, Madison, WI, gb:CVU471199). Among other features, pCI contains a CMV immediate early promoter-enhancer region, an optimized chimeric intron, a multiple cloning site (MCS), and the SV40 late polyadenylation signal sequence.

Starting with the plasmid pCI, a universal expression vector pCMV-MCS3 with three independent multiple cloning sites can be constructed as demonstrated in Fig. 1. This vector is suitable for individual in-frame cloning of, e.g., an ER-targeting leader sequence plus the gene of interest plus a heterologous helper epitope, and even allows the simple construction of dicistronic expression plasmids co-expressing an antigen and a cytokine via an internal ribosomal entry site (IRES).

Two long synthetic oligonucleotides are synthesized that span either the 5' part or the 3' part of the new MCS. 20 nucleotides at the 3' portion of each primer anneal to Art v 1 cDNA (gb:F493943), which also can serve as dummy antigen and may be replaced according to individual needs.

The long upstream primer MCS3 fw 5' *gctagcctcgag ATGGATGCAATGAAGAGAGGGCTCTGCTGTG TGCTGCTGCTGTGTGGAGCAGTCTTCGTTTCG GCAgtcgacgatatcgcatgcgaattcatggtggttcaaagtgtg* 3' contains the unique restriction sites for *NheI* and *XhoI* and the human plasminogen activator leader sequence (hTPA) followed by several restriction sites to facilitate cloning of the gene of interest. Restriction sites are underlined, the leader sequence is written in capitals, and the cleavage site of the signal peptide is marked in bold. The Art v 1-specific sequence is printed in italics.

The downstream primer MCS3 rv 5' *gcgccgc ttatctagaatgcattggccTTCTAAATGACTTGCACTTA CTTTGGTACTCTTAACCAGAAATTTACTGTAA AGTTATTAAAtcgatagcgt cccgggtgagtgagcgaggga ggtg* 3' incorporates a stop codon, singular restriction sites for *NarI*, *XbaI*, and *NotI*, and a 63 bp long tetanus

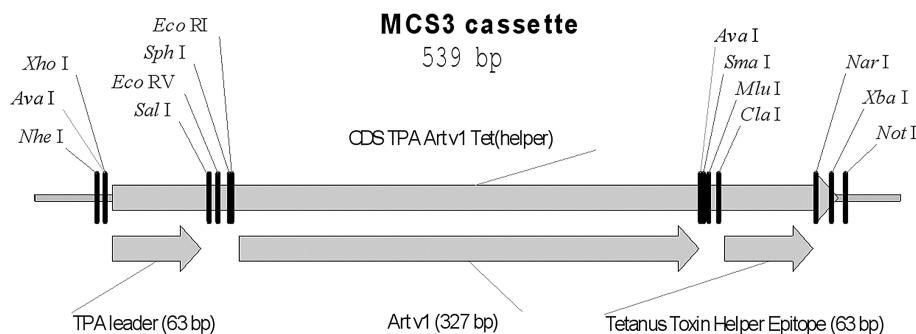


Fig. 1. A versatile universal expression vector—pCMV-MCS3. This vector allows cloning of a gene of interest (in this case the gene of the mugwort allergen Art v 1) into an MCS. If desired, the ER-targeting leader sequence (TPA) and/or the tetanus toxin helper epitope can be used, otherwise they can be removed by an *XhoI/SalI* or *ClaI/NarI* digest, respectively.

toxin helper epitope (fnnftvsfwlrpvksashle) followed by several unique restriction sites for subcloning a desired antigen (font types for restriction sites, etc., see above).

Inclusion of “foreign” helper epitopes has been shown to increase immunogenicity of weak antigens and may therefore represent an interesting approach for enhancing immune responses of suboptimal DNA vaccines [34,35].

Art v 1 cDNA serves as template for PCR and the 539 bp product is cut with *NheI* and *NotI* and ligated into the pCI mammalian expression vector. The resulting plasmid is designated pCMV-MCS3 and represents a useful tool for generating a variety of sophisticated plasmid DNA vaccines that include three different components. *XhoI* and *SalI* restriction enzymes generate compatible ends thus allowing removal of the leader sequence in one restriction digest step followed by intramolecular ligation of the remaining plasmid. A comparable feature is included in the downstream part of the molecule, where the tetanus toxin helper epitope can be removed in one step by digesting with the compatible enzymes *NarI* and *ClaI*. The additional stop codon between *XbaI* and *NotI* guarantees correct termination of translation of the transcribed mRNA.

2.1.2. DNA preparation

Large scale purification of the expression vectors is conducted with Endo Free Plasmid Giga kits (Qiagen, Hilden, Germany). Regenerating the column and reapplication can increase the plasmid yield 2- to 4-fold and significantly reduce the expenses in DNA vaccine research [36].

The transfected bacteria are plated on LB agar containing the recommended concentration of antibiotics and incubated for 24 h at 37 °C. Six characteristic colonies are picked with a pipette tip, inoculated separately into 4 ml LB broth, and incubated overnight at 37 °C. Mini-preps of plasmid DNA are then conducted from these samples and 1 ml of the culture showing the highest yield is selected. As much as 0.5 liter volumes of LB (total 2.5 liters) of the selected culture is incubated overnight at 37 °C in an appropriate bacterial shaker. The bacterial lysate is prepared and passed through the Qiagen column according to manufacturer's instructions but retaining the flowthrough. After eluting the plasmid according to the protocol, the column is washed with 100 ml sterile distilled water followed by 50 ml equilibration buffer. The retained flowthrough is now run a second time on the regenerated column. For re-use, 50 ml equilibration buffer is applied to the column, which is then sealed with parafilm and stored at 4 °C for later use with 2.5 liters of bacteria culture of the same plasmid.

Plasmid DNA is then analyzed by agarose gel electrophoresis, quantified by spectrophotometry (OD260/OD280 ratio >1.8), and stored in endotoxin-free H₂O at -20 °C.

2.1.3. Control for endotoxin contamination

The *Limulus* amoebocyte assay is a convenient and well-accepted method for measuring endotoxin (LPS of gram-negative bacteria) contamination in plasmid preparations and recombinant proteins. Every batch of plasmid DNA and protein should be measured and scored negative (Endotoxin <0.1 EU/μg plasmid) before immunizing animals. Synthetic ODNs also have to be tested for endotoxin to allow clear interpretation of their immunostimulatory effects.

Endotoxin is quantified with an endpoint turbidimetric LAL (*Limulus* amoebocyte lysate) method by measuring the level of turbidity (Pyrotell-T LAL kit, Pyroquant Diagnostik, Moerfelden, Germany).

Lyophilized LAL-reagent is reconstituted with LAL-reagent water (ACILA, Walldorf, Germany). A standard curve is generated from Standard Endotoxin NP-4 (Pyroquant Diagnostik, Moerfelden, Germany) diluted in LAL-reagent water ranging from 1.0 to 0.0625 EU/ml. Curves should also be constructed using endotoxin plus control DNA to rule out synergistic effects. Endotoxin-free LAL-reagent water serves as negative control.

In a clear flat-bottomed 96-well microtiter plate, 50 μl LAL-reagent is added to 50 μl sample as rapidly as possible and mixed on a plate shaker for 30 s. After 30 min incubation at 37 °C, the optical density (OD 405 nm) is determined in a plate reader. The incubation period depends on the endotoxin concentration range desired, so preliminary tests may be necessary to establish the correct incubation period; plate readers with kinetic measurement function can greatly simplify this procedure.

The mean optical density of the negative control is subtracted from all other readings and endotoxin concentrations are calculated from a standard curve by plotting optical density readings against standard endotoxin concentrations.

2.2. Immunization of animals

2.2.1. Intradermal immunization with plasmid DNA

Because of the great number of professional APCs (Langerhans cells), skin is the most effective target for inducing strong immune responses with DNA vaccines in mice. We commonly use female, 6–12 week old BALB/c, C57BL/6 or C3H/HeJ mice. However, due to their genetic disposition for developing Th2-biased immune responses, BALB/c mice are more frequently used in allergy model systems.

The preparation of the plasmid DNA vaccine does not require any chemical procedure or heating. Anesthetized mice are 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. I.d. injections are applied to several (i.e., 4–8) sites. The maximum injection volume is 30–50 μl per site.

Following an i.d. injection, a blister should persist in the skin for at least several minutes. If it disappears quickly or never forms, a subcutaneous injection has been given, which is likely not to work.

2.2.2. Subcutaneous and intraperitoneal sensitization with allergens

Mice are sensitized by three subcutaneous (s.c.) or intraperitoneal (i.p.) injections (days 0, 14, and 28) with 5 µg recombinant allergen adsorbed to 100 µl Al(OH)₃ (Serva, Heidelberg, Germany, 2 mg/ml) in a total volume of 150 µl sterile PBS. The adjuvant–protein complex is formed by mild shaking for at least 1-h before immunization. S.c. injections are given at two sites at the back, i.p. injections into one. There is no difference in strength, dynamics, and persistence of Th2-immune responses induced by s.c. or i.p. immunization with allergens. S.c. immunizations are easier to perform for untrained researchers.

2.3. Evaluation of the immune response

2.3.1. Collection of biological samples

Blood samples for preparation of sera are taken from the tail vein according to conventional techniques, incubated for 1 h at 37 °C for coagulation, and centrifuged for 20 min. Sera are transferred into fresh tubes and stored at 4 °C. Serum samples have to be collected before each immunization or challenge and about 2 weeks thereafter.

Spleens are collected at the end of the experiment, when mice are sacrificed.

2.3.2. Analysis of antigen-specific antibody production by enzyme linked immunosorbent assay

Black 96-well high-bind, flat-bottomed immunoplates (Greiner, Kremsmuenster, Austria) are coated by overnight incubation at 4 °C with recombinant antigen at a concentration of 1 µg/ml in PBS, pH 7.5, in a volume of 50 µl/well. Plates are washed three times with PBS/0.1% Tween 20 using the 96PW automatic enzyme linked immunosorbent assay (ELISA)-plate washing device (Tecan, Salzburg, Austria) and blocked with 200 µl/well blocking buffer (PBS, 0.1% (v/v) Tween 20, and 2% (w/v) skim milk, pH 7.5) for 1 h at room temperature. After washing three times, wells are incubated with individual serum samples (50 µl/well), serially diluted in blocking buffer, for 1 h at room temperature. Serum samples are usually tested starting at a dilution of 1:100, while high titered sera often require dilutions of up to 1:10,000. On each plate, 10 wells of a serially diluted standard serum (starting from 1:100) are added.

After washing five times, 50 µl/well of horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA), goat anti-mouse IgG1 (Serotec, Oxford, UK), rat anti-mouse IgG2a (PharMingen, San Diego,

CA), or rat anti-mouse IgE (Serotec, Oxford, UK) detection antibody is added in blocking buffer (1:1000) and incubated for 1 h at room temperature.

After washing five times, the assay is developed with 50 µl/well Luminol (BM chemiluminescence substrate, Boehringer–Mannheim, Germany) containing 0.1% hydrogen peroxide, diluted 1:2 in H₂O. Chemiluminescence (photon counts/second) is determined using a Lucy I Elisa-plate Luminometer (Anthos Labtec, Salzburg, Austria).

To compensate for plate to plate variations, a standard serum is prepared from pooled high titered antisera against the respective antigen. The standard serum is stored in 50% (v/v) glycerol, 0.2% (w/v) NaN₃ at –20 °C. For end-point titer determinations of the standard serum any well with a luminescence greater than 3 standard deviations above background (calculated using >20 wells containing no primary Ab) is scored as positive. The end-point titer of individual serum antibody bound to the plates can then be determined by interpolating into a standard curve generated with known dilutions of the standard serum.

2.3.3. Antigen-specific proliferative responses in splenocyte cultures

At the end of the experimental schedule, mice are sacrificed and spleens are aseptically removed. Spleens are minced in 1 ml culture medium (MEM) and aggregated cells are sedimented for 10 min. The supernatants containing a single cell suspension are washed in 10 ml MEM, centrifuged for 5 min at 300g, and re-suspended in 1 ml MEM supplemented with 1% (v/v) heat-inactivated fetal calf serum, 100 U penicillin, and streptomycin/ml, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 µM of 2-mercaptoethanol, 20 mM Hepes, and non-essential amino acids. Fifty microliters of this splenocyte solution is distributed into 96-well, flat-bottomed tissue culture plates (Becton–Dickinson, Franklin Lakes, NJ) at a density of 10⁶ cells/well. Recombinant antigen is added in 50 µl supplemented culture medium for a final concentration of 20 µg/ml to five wells/spleen and plates are incubated for 72 h at 37 °C, 95% relative humidity, 7.5% CO₂. Five replicate wells stimulated with an irrelevant antigen and with medium alone serve as negative controls; wells stimulated with a final concentration of 0.25 µg/ml of the mitogen concanavalin A are used as positive control. After 3 days, wells are pulsed with 0.5 µCi [³H]thymidine added in a volume of 20 µl culture medium for another 20 h. Then, the cells are harvested with a cell harvester (Cell Harvester 96 Mach IIIM, Tomtec, CT, US). Thymidine incorporation is measured in a scintillation counter (Wallac MicroBeta TriLux, Perkin–Elmer, Finland). Stimulation indices are calculated from the averages of five replicate wells according to the equation, [antigen_{cpm}/medium_{cpm}]. Additional wells stimulated with recombinant antigen and wells

with culture medium alone are cultured for the evaluation of cytokine production *in vitro*. Supernatants of these wells are taken after 4 days of incubation, which can be stored at -70°C for later examination.

2.3.4. Quantification of cytokines in supernatants of re-stimulated splenocytes

IFN- γ and IL-5 in supernatants of re-stimulated splenocytes are quantified by sandwich ELISA using the OptEIA system (PharMingen, San Diego, CA). Black 96-well high-bind, flat-bottomed immunoplates (Greiner, Kremsmuenster, Austria) are coated by overnight incubation at 4°C with monoclonal anti-mouse IFN- γ /IL-5 capture antibodies diluted 1:250 in coating buffer (0.1 M carbonate, pH 9.5, freshly prepared) in a volume of 50 μl /well. After washing three times with PBS, 0.1% (v/v) Tween 20, plates are blocked with 200 μl /well PBS, 0.5% (v/v) Tween 20 for 1 h at room temperature. Plates are washed three times and 50 μl /well of supernatants diluted 1:2 in PBS, 1% (w/v) BSA is added and incubated for 1 h at room temperature. A standard curve is generated from recombinant murine IFN- γ /IL-5 (PharMingen, San Diego, CA) diluted in culture medium/PBS, 1% (w/v) BSA (1:2) ranging from 5000 to 78 pg/ml (IFN- γ) or 500 to 7.8 pg/ml (IL-5), respectively. After washing five times, captured cytokine is detected by adding 50 μl /well of working detector. Working detector is composed of biotinylated IFN- γ /IL-5 detection antibody diluted 1:500 in culture medium/PBS, 1% (w/v) BSA (1:2) and enzyme reagent (avidin–HRP) diluted 1:250. After 1 h incubation at room temperature, plates are washed five times and the luminometric assay is developed as described above. IFN- γ /IL-5 is then quantified by interpolation into the standard curve. A highly sensitive and elegant alternative to the ELISA technique is the simultaneous quantification of a panel of cytokines from culture supernatants by the Fluorescent Bead Immunoassay (Bender MedSystems, Vienna, Austria) on a Luminex100 LapMap System (Luminex, Austin, TX).

2.3.5. β -Hexosaminidase release from rat basophil leukemia cells

Together with other mediators such as histamine, β -hexosaminidase is an enzyme, which is released during degranulation of mast cells and basophils. Therefore, the rat basophil leukemia (RBL) cell release assay provides a functional read-out for IgE-mediated degranulation.

RBL-2H3 cells (ATCC Nr. CRL-2256) are distributed into 96-well, flat-bottomed tissue culture plates (Becton–Dickinson, Franklin Lakes, NJ) at a density of 4×10^4 cells/well in 100 μl of RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U penicillin and streptomycin/ml, 4 mM L-glutamine, 2 mM sodium pyruvate, 10 mM Hepes, and 100 μM of

2-mercaptoethanol. Plates are incubated overnight at 37°C , 95% relative humidity, 7% CO_2 .

For passive sensitization of RBL cells, 50 μl of supernatant is removed and serum from immunized and control mice added at a final concentration of 1:30. Background wells and wells for maximal release are left untreated. Plates are incubated for 2 h at 37°C , 95% relative humidity, 7% CO_2 . To remove unbound antibodies, cells are washed twice with 200 μl Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl_2 , 1.8 mM CaCl_2 , 0.4 mM NaH_2PO_4 , 5.6 mM D-glucose, 12 mM NaHCO_3 , 10 mM Hepes, and 0.1% (w/v) BSA, pH 7.2). Crosslinking of Fc ϵ R-bound IgE and subsequent degranulation of RBL cells is induced via addition of 100 μl of a 0.3 $\mu\text{g}/\text{ml}$ solution of recombinant allergen or 100 μl pollen extract ($c = 1.5 \mu\text{g}/\text{ml}$) in Tyrode's buffer. Cells are incubated for 30 min at 37°C , 95% relative humidity, 7% CO_2 . For determination of maximal release, 10 μl of a 10% Triton X-100 solution is added to some wells and re-suspended. Plates are then centrifuged at room temperature for 5 min at 300g and 50 μl of supernatants are transferred to fresh plates, which can be frozen at -20°C for later examination.

4-Methyl umbelliferyl-*N*-acetyl- β -D-glucosaminide (4-MUG) is a substrate for β -hexosaminidase and forms stable complexes with this enzyme, which can be detected by fluorescence spectroscopy. Analysis of β -hexosaminidase activity is performed by incubation of 50 μl supernatant with 50 μl 4-MUG (Sigma, Deisenhofen, Germany) in citrate buffer (0.1 M, pH 4.5) at a final concentration of 80 μM for 1 h at 37°C , 95% relative humidity, 7% CO_2 . The reaction is stopped via adding 100 μl glycine buffer (0.2 M glycine and 0.2 M NaCl, pH 10.7) and fluorescence (in relative fluorescence units) is measured at λ_{ex} (360 nm)/ λ_{em} (465 nm) using a fluorescence microplate reader (Spectrafluor, Tecan, Austria). Results are calculated according to the equation, $[(\text{experimental}_{\text{rfu}} - \text{background}_{\text{rfu}})/(\text{maximum}_{\text{rfu}} - \text{background}_{\text{rfu}})] \times 100$.

2.3.6. Passive cutaneous anaphylaxis assay

IgG1 and IgE are the only Ig isotypes, which can elicit active and passive anaphylactic reactions in mice through binding to Fc γ RIII and Fc ϵ RI, respectively, on mast cells [37]. Anaphylactic activity of sera obtained from immunized mice is determined by passive cutaneous anaphylaxis reactions in naïve animals.

Mice are shaved on the back and three intradermal injections (20–30 μl) of serial dilutions of sera in PBS are made on each side of the dorsal skin. After 2 h, mice are challenged with 200 μl of 0.5% (w/v) Evans blue solution containing 250 μg antigen via intravenous injection at the tail. Ten minutes later, the animals are sacrificed and the dorsal skin is carefully removed. PCA titers are expressed as the reciprocal of the highest dilution, which gives a blue lesion of 5 mm in diameter. All PCAs have to be performed at least

in duplicate. As the ability of IgE to bind to FcεRI is lost after heating, passive cutaneous reactions induced by sera inactivated at 56°C for 1 h will be only due to IgG1 antibodies.

2.4. Modification of translation

2.4.1. Recoding of allergens

Genetic immunization offers an enormous palette of modulating possibilities to influence the immunogenicity of plasmid constructs [5]. 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, suboptimal codons of a heterologous gene can have a drastic effect on protein expression by the transfected cell with significant influence on the immunogenicity of a DNA vaccine [38–40].

A gene vaccine including the wild type sequence for Art v 1 fails to be immunogenic in mice (unpublished results). Recoding of the vaccine with a synthetic gene sequence based on the human codon usage increases the expression level of the antigen and enhances the immunogenicity of the construct.

The “humanized” gene is synthesized in a template-free PCR using three overlapping oligonucleotides (125-mer, 128-mer, and 147-mer) spanning the entire Art v 1 sequence.

Flanking restriction enzyme sites for subcloning and 25 bp overlaps are included in the oligonucleotides to ensure efficient annealing of the individual primers.

Codons are adapted according to the codon usage tabulated from GenBank (<http://www.kazusa.or.jp/codon>) for the optimal human codon preference [41] as demonstrated in Table 1. No deviations from strict adherence to optimized *Homo sapiens* codon usage are accepted.

The 350 bp fragment generated in the recoding process of Art v 1 is ligated into an *EcoRI*–*XbaI* digested pCMV-MCS3 expression vector. The resulting plasmid encodes Art v 1 by using the most frequent codons commonly found in highly expressed human genes (Table 1).

2.4.2. Increasing MHC class II presentation by addition of a eukaryotic leader sequence

The use of an ER-targeting leader sequence not only enhances humoral immunity, but also cellular responses. By circumvention of cytosolic translation (and possibly degradation) the amount of secreted native protein, which is a major prerequisite for antigen presentation by APCs, is increased [5].

Vector pCMV-MCS3 (Fig. 1) contains the first 63 bp of the hTPA signal sequence followed by several restriction sites to facilitate cloning of the gene of interest. At the junction between the leader sequence

and the multiple cloning site, the codon GCA, coding for Ala, has been added facilitating cleavage of the signal peptide according to the signal peptide prediction algorithms of Nielsen et al. [42] and Nielsen and Krogh [43].

2.4.3. Creating non-anaphylactic and safe DNA vaccines via ubiquitination

To avoid cross-linking of pre-existing IgE antibodies with the allergen by the DNA vaccine (thus inducing mast cell release), translation can be guided to the proteasome by fusing the gene of interest with ubiquitin. Cytoplasmic proteins are degraded by the ubiquitin-dependent pathway through the action of proteasomes and the resulting peptides are then transported by the Tap1/Tap2 heterodimer into the ER, where they associate with MHC-I molecules and β2-microglobulin. The resulting complexes are then transported to the cell surface, where they are recognized by CD8⁺ T-cells. Following DNA immunization, only a small percentage of plasmid encoded proteins becomes ubiquitinated. It has been demonstrated that stably linking ubiquitin to the antigen of interest via a covalent bond efficiently channels the ubiquitinated protein into the polyubiquitination pathway and to the proteasome, thereby shifting the immune response against the encoded antigen towards a Th1-type and simultaneously decreasing or even abolishing antibody reactions while enhancing the cytotoxic response [44–47].

To accomplish this, a ubiquitination vector has been constructed in the following way:

The ubiquitin gene has been amplified by PCR from genomic DNA of C57/BL6 mice with the following primers: *NheI*-Ubi fw (CCCGCTAGCCACCATGCAGATCTTCGTGAAGACCCTG) and Ubi-*EcoRI* rv (CCGAATTTCGGCACCTCTCAGGCGAAGGACCAGG) containing the respective restriction sites (*italics*). Additionally, the forward primer contains a Kozak's consensus sequence (*underlined*) to rule out dual initiation of translation at a second AUG codon. The PCR fragment has been digested with *NheI* and *EcoRI* and cloned into vector pCI (Promega, Madison, WI), which has been digested with the same enzymes. The 3' primer changed the C-terminal amino acid from Gly (G₇₆) to Ala (A₇₆), which makes ubiquitin relatively resistant to cleavage, thus targeting the stably ubiquitinated protein into the polyubiquitination pathway more efficiently [45]. Downstream of the ubiquitin sequence, the MCS of vector pCI contains several restriction sites that allow easy in-frame insertion of a gene of interest.

2.5. Co-expression of antigens, cytokines or co-stimulatory factors

Co-delivering genes for immunologically important molecules, such as cytokines and co-stimulatory

Table 1
Recoding of the Art v 1 gene

Aminoacid	Art v 1 codons	<i>n</i> for Art v 1 ^a	Human codon frequency/1000 ^b	Optimized codon ^c	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		
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		
	AGC	1	19.3		
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

^a The number of the respective codons as used in the wild type Art v 1.

^b Codon frequencies of highly expressed human genes (human codon frequency per thousand).

^c The codon-optimized Art v 1 gene always uses the optimal codon concerning the human codon usage (expressed for all optimized codons in the rightmost column).

molecules, are a unique possibility of genetic vaccines and provide fine tuning of the magnitude and direction (humoral and cellular) of the immune responses induced [5].

Two different approaches can be utilized to insert molecular-adjuvant genes into an expression vector: (i) cloning different inserts into a single vector under the

control of independent promoters or (ii) constructing polycistronic expression plasmids in which different proteins are translated from a single mRNA species. Expression of different cistrons in polycistronic expression plasmids is strictly coupled and protein expression of the different genes will not vary.

Both strategies are advantageous over mixing combinations of antigen expressing and modulating plasmids because they guarantee a constant expression of both products in the transfected cells.

2.5.1. Construction of a vector expressing two genes under the control of different promoters

Two new and unique restriction sites *Bst*WI and *Sac*II are introduced into the commercially available plasmid pCI-neo (Promega, Madison, WI, gb:CVU47120). This vector contains all the beneficial structure elements of the pCI expression vector and the possibility to exchange the original SV40 promoter controlled neomycin sequence against an immunomodulatory gene of interest. The new restriction enzyme sites are introduced with mutagenic oligonucleotide primers

using Strategene's QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) flanking the neomycin resistance gene. Both mutagenic primers contain the desired mutations and anneal to the same sequence on opposite strands of the original pCI-neo plasmid.

For introducing the upstream *Bst*WI site the upper primer 5' AACAGTCTCGAAC**CTACGG**CTAGAGC CACC 3' and the lower primer 5' GGTGGCTCTAGC **CGTACG**TTCGAGACTGTT 3' are used.

The new *Sac*II site downstream of the neomycin-gene is generated with the upper primer 5' TCTGAGCGG GAC**CGCGGGG**TTTCGAAATGAC 3' and the lower primer 5' TCATTTCGAAC**CCCCGCGG**TCCCGCT CAGA 3'. The mutated nucleotides are written in bold and underlined. In vitro site-directed mutagenesis is carried out according to manufacturer's instructions. The two new and unique restriction enzyme sites in the resulting vector enable us to replace the neomycin gene with any modulatory- or antigen gene of interest (Fig. 2). The SV40 promoter driving this second expression cassette works with a similar efficiency as the CMV promoter in the first cassette (unpublished data).

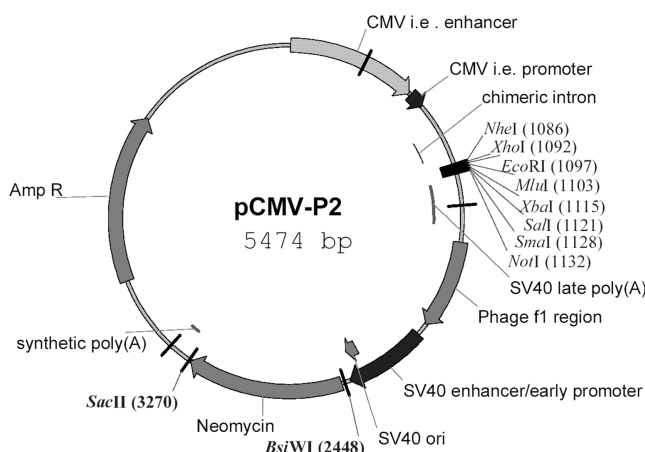


Fig. 2. A double-promoter vector for the co-expression of genes—pCMV-P2. A CMV promoter drives transcription of a gene of interest cloned into the MCS. Additionally, an SV40 promoter initiates transcription of a neomycin resistance gene. By digesting this vector with *Bst*WI and *Sac*II, the neomycin cassette can be removed and substituted for another gene, e.g., a cytokine.

2.5.2. Construction of a vector expressing two genes under the control of the same promoter

An IRES was used to enable the translation from a dicistronic mRNA under the control of the same promoter. The vector pCMV-MCS3 (see above) is already prepared to incorporate the structure “promoter-geneA-IRES-geneB” as demonstrated in Fig. 3.

pIRESHyg2 (Clontech, Palo Alto, CA, order# PT3411-5) serves as a source of an IRES sequence that originates from encephalomyocarditis virus (EMCV). PCR is conducted with the primers IRES fw 5' CACCGAATTCGAGCATGCATCTAGGGCGGC 3' incorporating an *Eco*RI site (italics) and IRES rv 5' GATCACGCGTTTAATCCATCTTTGGTACGGGTTGTG 3' incorporating a *Mlu*I site (italics). The resulting 615 bp long amplicon is ligated into the *Eco*RI–*Mlu*I sites of pCMV-MCS3. The up- and downstream of the IRES-motif located restriction enzyme sites offer cloning of two different genes both under the control of the CMV promoter in pCMV-MCS3 (Fig. 3).

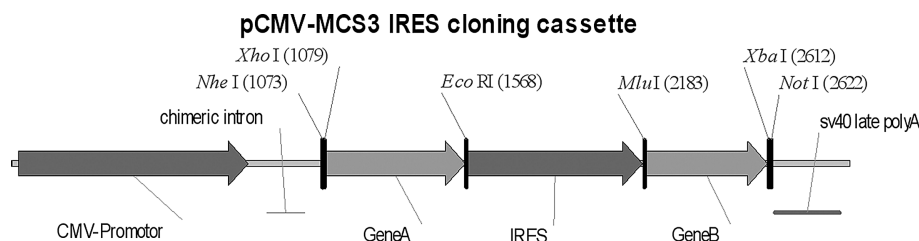


Fig. 3. A vector expressing two genes with one promoter—pCMV-MCS3 IRES. This vector initiates transcription of a bicistronic mRNA, two different genes (Gene A and Gene B) are separated by an IRES site that allows translation of two different proteins from a single mRNA.

Fig. 4. Appending of CpG-rich sequences. The amino acids are encoded by a DNA sequence, which contains four immunostimulatory CpG motifs. The necessary stop codon is indicated by asterisks.

We therefore recommend including both, a protective and a therapeutic approach in every experimental design for the characterization of DNA-based immune responses. A reliable standard protocol for the first experiments using a protective approach could be: three i.d. injections of plasmid DNA in weekly intervals followed by s.c. or i.p. sensitization with antigen between 30 and 50 days after the last DNA immunization. The same time schedule can be used for a therapeutic approach vice versa, with antigen sensitization followed by DNA treatment.

Control experiments with a vector lacking the insert or a vector encoding an irrelevant antigen are an absolute requirement, because the immunostimulatory CpG effect of plasmid DNA can overrule the antigen-specific effect of DNA immunization (within a certain window of time—depending on the antigen and/or the immunization schedule) and lead to antigen-independent protective and therapeutic effects [48].

In general, DNA vaccines encoding allergens are immunogenic without any further modification such as secretion signals, recoding or co-stimulation. The reason for this may be the high degree of solubility, a special property of allergens. In many cases, however, these vaccines are still suboptimal as can be demonstrated by insertion of a leader sequence to the gene of Bet v 1a, which increases the immunogenicity further and enhances the functional efficiency (unpublished data). Therefore, we recommend to clone and test several versions of DNA vaccines for each allergen gene (e.g., a simple form encoding only the allergen, a construct with leader, and/or heterologous helper epitope, a recoded and a CpGized version and if desired, constructs co-expressing certain immunomodulatory stimuli).

With respect to its Th1-inducing capacity, DNA immunization offers highly interesting approaches to develop safe vaccines, which should not cause any anaphylactic side effect because of lacking the production of native allergen (the target of IgE-cross-linking on mast cells) during the course of immune therapy. This can be managed by cutting the allergen into non-anaphylactic fragments, ubiquitination or CpGizing.

Fragmentation represents a very simple approach; however, the fragments must be controlled concerning their loss of three-dimensional structure and native epitopes, respectively.

Ubiquitination with a vector as proposed above can solve these problems, but it must be stated, that at present Th-activation after ubiquitination is not fully understood.

CpGizing represents a third alternative to reduce the amount of translated native allergen and is of comparable efficiency with respect to the functional read-outs but the induced immune responses should be carefully

monitored concerning possible negative effects of excessively Th1-biased immune responses.

Summing up, DNA immunization offers an enormous potential to break new ground in vaccine research and to develop alternative immunotherapeutic approaches for the treatment of allergic diseases.

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