

Generation of hypoallergenic DNA vaccines by forced ubiquitination: Preventive and therapeutic effects in a mouse model of allergy

Roman Bauer, PhD,^{a*} Sandra Scheiblhofer, PhD,^{a*} Kerstin Kern, MSc,^a Christina Gruber, MSc,^a Tatjana Stepanoska, PhD,^a Theresa Thalhamer, MSc,^a Cornelia Hauser-Kronberger, PhD,^b Beate Alinger, MSc,^b Thomas Zoegg, MSc,^a Maximilian Gabler, PhD,^a Fatima Ferreira, PhD,^c Arnulf Hartl, PhD,^d Josef Thalhamer, PhD,^a and Richard Weiss, PhD^a Salzburg, Austria

Background: Hypoallergenic immunotherapy of type I allergies aims at inducing T-cell immunity while avoiding cross-linking of pre-existing IgE. DNA-based immunotherapy depends on the recruitment of antigen-specific T_H1 cells and therefore has to provide the whole repertoire of T-cell epitopes. Ubiquitination offers a general approach for the production of hypoallergenic DNA vaccines.

Objective: A DNA-based vaccine encoding the major birch pollen allergen Bet v 1 stably linked to ubiquitin was evaluated for its antiallergic potential in a BALB/c mouse model of allergy.

Methods: Plasmid DNA was applied to mice before (preventive) or after (therapeutic) sensitization with recombinant Bet v 1. In the preventive setting, mice were exposed to aerosolized allergen in addition. Cytokine production was monitored via ELISPOT and Luminex. IgG₁, IgG_{2a}, and IgE subclass antibody titers were determined by ELISA. *In vitro* antigen-specific cross-linking of IgE was measured in a degranulation assay. Bronchoalveolar lavages were analyzed for leukocyte subsets as well as for IFN- γ and IL-5, and paraffin sections of lungs were examined for mucus production and endothelial damage.

Results: Prevacination with ubiquitinated Bet v 1-stimulated T_H1-biased immune responses with concomitant suppression

of functional IgE, reduction of eosinophil counts in bronchoalveolar lavages, and alleviation of lung pathology, and could also suppress an ongoing IgE response in a therapeutic setting.

Conclusion: The data clearly demonstrate that hypoallergenic DNA vaccines encoding ubiquitin fusion constructs induce effective antiallergic immune responses.

Clinical implications: Ubiquitination of allergen gene vaccines eliminates the risk of IgE cross-linking, thereby meeting the safety requirements for clinical applications. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Type I allergy, DNA vaccine, genetic immunization, gene therapy, ubiquitin, IgE, bronchoalveolar lavage, eosinophils, lung pathology

Specific immunotherapy (SIT) using crude extracts of pollen harbors several disadvantages, including lack of standardization, frequent occurrence of anaphylactic reactions caused by cross-linking of pre-existing IgE antibodies on mast cells, and an initial rise in allergen-specific IgE.¹ Moreover, the use of complex allergen mixtures also implies the risk of cross-sensitization against allergenic components to which the individual has not been sensitized before.^{2,3} Therefore, future SIT may be based on component-resolved diagnosis and the use of defined allergen molecules produced by recombinant DNA technology or synthetic and genetically modified hypoallergenic derivatives (see review⁴).

DNA-based desensitization methods, as an alternative to SIT with protein, will be submitted to the same requirements, especially concerning the hypoallergenicity of the vaccines. In the past years, intramuscular or intradermal injection of allergen-encoding plasmid DNA has been shown to induce a T_H1-biased immune response characterized by promotion of IFN- γ -producing CD4⁺ T cells, elevated production of IgG_{2a}, and inhibition of IgE. Numerous animal studies applying this new immunotherapeutic technique demonstrated its efficacy.⁵⁻¹²

Genetic desensitization avoids the risks associated with conventional immunotherapy for several reasons. After

From ^athe Department of Molecular Biology, Division of Allergy and Immunology, ^bthe Department of Pathology, Paracelsus Private Medical University; ^cthe Christian Doppler Laboratory for Allergy Diagnostic and Therapy; and ^dthe Institute of Physiology and Pathophysiology, Paracelsus Private Medical University, Salzburg.

*These authors contributed equally to this work.

Supported by the Austrian Science Foundation, project # S8811, S8813, S8802, T133-B08, and the Ludwig Boltzmann Institute for Experimental Surgery.

Disclosure of potential conflict of interest. The authors have declared that they have no conflict of interest.

Received for publication February 1, 2006; revised March 21, 2006; accepted for publication March 22, 2006.

Reprint requests: Josef Thalhamer, PhD, University of Salzburg, Department of Molecular Biology, Division of Allergy and Immunology, Hellbrunnerstrasse 34, 5020 Salzburg, Austria. E-mail: Josef.Thalhamer@sbg.ac.at.

0091-6749/\$32.00

© 2006 American Academy of Allergy, Asthma and Immunology

doi:10.1016/j.jaci.2006.03.033

Abbreviations used

BAL:	Bronchoalveolar lavage
pcDNA Luc:	Plasmid vector expressing firefly luciferase
pCMV:	Empty plasmid vector
pCMV-β:	Plasmid vector expressing β-galactosidase
pCMV-Bet:	Plasmid vector expressing Bet v 1a
pCMV-ubi-β:	Plasmid vector expressing ubiquitinated β-galactosidase
pCMV-ubi-Bet:	Plasmid vector expressing ubiquitinated Bet v 1a
RBL:	Rat basophil leukemia
SIT:	Specific immunotherapy

DNA-based immunization, the amount of translated antigen is at least 3 to 5 orders of magnitude lower than the amount of protein injected during the course of SIT. Because of the T_H1-biased immune response induced via intradermal or intramuscular application of plasmid DNA, no *de novo* synthesis of IgE occurs. Furthermore, in contrast with SIT using hardly standardized crude extracts, DNA vaccines express defined allergens of high purity.

Nevertheless, the high safety profile required for entering clinical trials affords the development of antiallergic DNA vaccines expressing hypoallergenic molecules. Translation of native allergenic determinants must be avoided while T-cell epitopes of the encoded allergen must not be destroyed to guarantee the recruitment of allergen-specific T_H1 cells. This was already achieved by fragmentation or deliberate mutation of the allergen gene¹³⁻¹⁵; however, such knowledge-based approaches are time-consuming, and their suitability must be tested on a case-by-case basis for individual allergens.

In contrast, ubiquitination of allergens represents a routine approach for destruction of IgE-binding epitopes to produce hypoallergenic DNA vaccines. Ubiquitin was initially proposed to target harmful cellular proteins to the disposal in the proteasome. Today, we know that monoubiquitination and polyubiquitination are involved in numerous regulatory processes in the cell, including modification of protein function, cell surface receptor turnover, control of gene transcription, and immune regulation (see review¹⁶). Degradation of cytoplasmic proteins via the ubiquitin-dependent pathway through the action of the proteasome results in presentation of antigenic peptides on MHC-I. After conventional DNA immunization, only a small percentage of plasmid-encoded protein becomes naturally 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, thereby shifting the immune response against the encoded antigen toward a T_H1 type and simultaneously decreasing or even abolishing antibody reactions and enhancing cytotoxic responses.¹⁷⁻²²

In our current study, we investigated the potency of a DNA-based vaccine encoding a ubiquitinated version of Bet v 1, the major allergen from birch pollen, to protect

mice from subsequent sensitization with the allergen or to convert established allergic immune responses. Our data show for the first time that ubiquitination of an allergen renders the molecule hypoallergenic—that is, DNA-based immunization induced no detectable antibody responses, but T-cell reactivity was completely preserved as indicated by antigen-specific proliferation, cytokine production, and, above all, protection from allergic reactions.

METHODS

Plasmids

Plasmid vector expressing Bet v 1a (vector pCMV-Bet) contains a single copy of the coding sequence of the major birch pollen allergen Bet v 1a and has been described previously.¹²

The sequence encoding ubiquitin was amplified from the mouse genome via PCR. As a template, genomic DNA was prepared from mouse spleen with a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to the protocol of the manufacturer. DNA was amplified with the sense primer cccGCTAGCcacca tgcagatctctgtgaagaccctg and the antisense primer cccGAATTCggcaccctctca ggcaag-gaccagg. The sense primer introduced a NheI site (*bold caps*) and a Kozak consensus sequence (*underlined*), which is crucial for efficient initiation of translation.²³ The antisense primer introduced an EcoRI site (*bold caps*) and a point mutation (gcc → ggc; anti-sense strand, *underlined*) that changed Gly76 to Ala76. This mutation diminishes the rate of cleavage of the fusion protein and thus enhances degradation of the protein.²⁴

A 250-bp fragment was amplified by PCR using Pfu polymerase (Promega, Mannheim, Germany) and cloned into the NheI/EcoRI restriction sites of pCMV-Bet, thereby generating the plasmid vector expressing ubiquitinated Bet v 1a (pCMV-ubi-Bet).

In vitro expression

To determine levels of protein expression from the respective vectors *in vitro*, transfections with 1, 0.5 or 0.25 μg plasmids expressing either β-galactosidase (pCMV-β) or β-galactosidase stably linked to ubiquitin (pCMV-ubi-β) were performed. Briefly, B16-F1 mouse melanoma cells were seeded at 0.8×10^5 per well of a 24-well plate 1 day before transfection using SuperFect (Quiagen, Hilden, Germany) according to the manufacturer's protocol. As an internal control for transfection efficacy, cells were cotransfected with 1 μg plasmid vector expressing firefly luciferase (pcDNA Luc)—plasmid, which was generated by cloning the luciferase gene from pGEM-luc (Promega) into pcDNA1 expression vector (Invitrogen, Lofer, Austria). Twenty-four hours after transfection, reporter activity was determined by Dual-Light combined reporter gene assay system (Applied Biosystems, Lincoln, Calif) using a Lucy II ELISA plate luminometer (Anthos Labtec, Wals, Austria).

Mice, immunization, and serology

Female BALB/c mice (n = 11; 6-8 weeks old; Charles River, Sulzfeld, Germany) were immunized intradermally 3 times at weekly intervals with 100 μg pCMV-Bet, pCMV-ubi-Bet, or empty mock vector (pCMV). Two weeks after the last immunization, mice were sensitized by 3 subcutaneous injections at 2-week intervals of 5 μg recombinant Bet v 1 (Biomay, Vienna, Austria) in sterile PBS with 100 μL alum (Al(OH)₃; Serva, Heidelberg, Germany) in a total volume of 200 μL into 2 spots on the back. Two weeks after the last sensitization, lung pathology was induced by exposure to aerosolized Bet v 1 (0.2 % in endotoxin-free PBS) for 20 minutes on 3 consecutive days using a PARI BOY N inhalation system (PARI, Starnberg,

Germany). Additional groups of mice ($n = 6$) were killed 2 weeks after the 3rd DNA immunization to assess the prechallenge immune status.

In a therapeutic setting, groups of mice ($n = 8$) were presensitized via 4 subcutaneous injections of Bet v 1 in alum as described at weeks 0, 2, 4, and 6. Mice received therapeutic intradermal injections of 100 μg pCMV-Bet, pCMV-ubi-Bet, or pCMV and were subsequently rechallenged by subcutaneous injections with recombinant allergen/alum. This was repeated twice (Fig 6, D). IgG₁, IgG_{2a}, and IgE antibody levels in sera taken at regular intervals were determined by a luminescence-based ELISA as described.²⁵

All animal experiments were conducted according to local guidelines approved by the Austrian Ministry of Science.

Lymphocyte cultures

Lymphoproliferation of splenocytes was measured as described¹³ (except that 1% FCS instead of 5% FCS was used, and cells were plated at a density of 2×10^5 cells per well). IFN- γ , IL-5, and IL-10 were measured in culture supernatants by Fluorescent Bead Immunoassay (Bender MedSystems, Vienna, Austria) on a Luminex100 LabMap System (Luminex, Austin, Tex) according to the manufacturer's instructions.

ELISPOT assay

Lymphocytes prepared as aforementioned were cultured in anti-IFN- γ -coated and anti-IL-5-coated (4 $\mu\text{g}/\text{mL}$) ELISPOT plates (Millipore, Vienna, Austria) with antigen for 24 hours as described for proliferation cultures. Cytokines were detected with biotinylated mAbs (2 $\mu\text{g}/\text{mL}$) followed by streptavidin-horseradish peroxidase (1:1000). Matched antibody pairs and streptavidin-horseradish peroxidase were purchased from Becton Dickinson Pharmingen (Schwechat, Austria). The assay was developed by using 3-amino-9-ethyl-carbazole substrate (Acros, Geel, Belgium).

β -Hexosaminidase release from rat basophil leukemia cells

As a functional read-out for IgE-mediated degranulation, a β -hexosaminidase release assay was performed by using the rat basophil leukemia (RBL)-2H3 cell line as described.¹³

Analysis of bronchoalveolar lavages

Bronchoalveolar lavages were performed with 2×1 mL fresh, ice-cold PBS using a hypodermic needle. Samples were centrifuged through a 40- μm cell strainer (Becton Dickinson). Erythrocytes were lysed for 10 minutes at room temperature with 500 μL FACS-Lyse (Becton Dickinson). Cells were washed in FACS-Buffer (PBS, 0.5% BSA, 2 mmol/L EDTA), resuspended in 1 mL FACS-Buffer, labeled with anti-CD45 FITC (1:400; Becton Dickinson Pharmingen) on ice for at least 10 minutes, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Eosinophils were distinguished from other leukocyte populations by their CD45^{med} side-scatter^{high} phenotype, and their relative amount compared with total CD45⁺ cells was calculated. Results from flow cytometry were confirmed by standard histologic analysis of cytospin preparations.

Lung histology

Lungs from 5 mice per group were fixed in 4% formalin at 4°C overnight and embedded in paraffin. Tissue sections of 2 μm were stained with periodic acid-Schiff using standard protocols. Immunostaining of the proliferation marker Ki-67 was performed with TEC-3 antibody (Dako, Vienna, Austria) using the streptavidin-biotin-peroxidase-complex method. Briefly, tissue sections were deparaffined and watered; endogenous peroxidases were blocked by using 3% H₂O₂-methanol. Tissue sections were then autoclaved in citrate

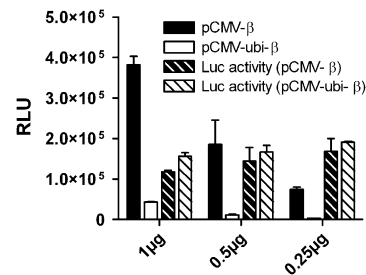


FIG 1. *In vitro* expression of β -galactosidase. B16-F1 cells were transfected with different amounts of pCMV- β or pCMV-ubi- β . Cells were cotransfected with equal amounts of a luciferase reporter construct as an internal control for transfection efficacy. Data are shown as averages $\pm$ SEMs of 2 individual transfections. Results are representative of 3 independent experiments.

buffer and incubated with rabbit serum. Primary antibody was diluted 1:25 in standard diluent (Dako) and incubated at 4°C overnight. Secondary biotinylated antibody was diluted in PBS (1:200) and incubated for 30 minutes. Slides were developed with diaminobenzidine and counterstained with hematoxylin/eosin dye.

All sections were analyzed by light microscopy. In periodic acid-Schiff sections, the number of goblet cells in bronchioles was determined to detect goblet cell metaplasia. Proliferating epithelial cells as markers for tissue damage in bronchioles and blood vessels were counted in TEC-3-stained sections.

Statistical analysis

Statistical differences between means of individual sample groups were assessed via the Student *t* test. Groups that failed normality test were analyzed by Mann-Whitney rank-sum test as indicated. *P* values of serially diluted serum samples from different groups were calculated by ANOVA.

RESULTS

In vitro expression of ubiquitinated antigen

To assess the efficacy of ubiquitination, B16-F1 cells were transfected *in vitro* with DNA vaccines encoding β -galactosidase or a ubiquitinated version of the molecule. As a control for transfection efficacy, cells were cotransfected with equal amounts of a luciferase reporter construct. β -Galactosidase activity in cells transfected with the vector encoding the ubiquitinated version of the enzyme was reduced by as much as 95% compared with cells transfected with a vector encoding the native enzyme at an equal transfection efficacy (Fig 1).

A DNA vaccine encoding ubiquitinated Bet v 1 induces no antibodies but stimulates substantial allergen-specific T-cell proliferation

In contrast with BALB/c mice immunized with pCMV-Bet, which showed high titers of IgG₁ and also IgG_{2a} antibodies, no significant antibody response could be measured after immunization with pCMV-ubi-Bet (Fig 2, A). However, splenocytes of these animals responded with strong proliferation after restimulation with recombinant Bet v 1 (Fig 2, B). The DNA vaccine coding for the

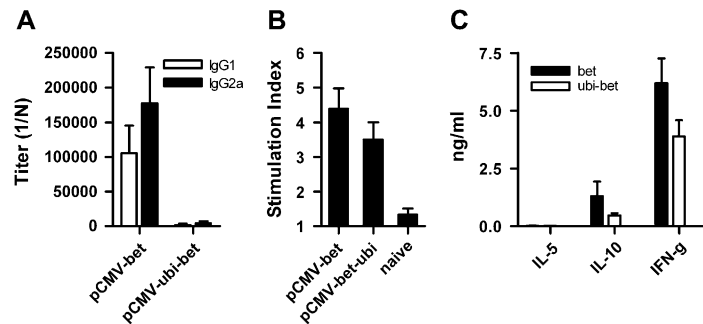


FIG 2. Characterization of immune responses. Antibody (A), proliferative (B), and cytokine (C) responses after immunization with pCMV-Bet compared with pCMV-ubi-Bet. Data are shown as means $\pm$ SEMs ($n = 6$). The experiment was repeated twice with similar results.

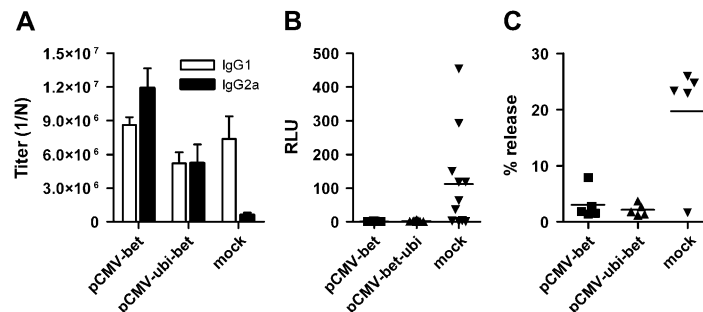


FIG 3. Antibody subclasses after sensitization with recombinant Bet v 1/alum. Bet v 1-specific IgG₁/IgG_{2a} (A) and IgE (B) were determined by ELISA, and biologically active IgE was measured by RBL release assay (C). Mice ($n = 11$) had been preimmunized with pCMV-Bet, pCMV-ubi-Bet, or empty vector (mock). Values are shown as means $\pm$ SEMs (A) or as sera from individual animals (B and C).

ubiquitinated gene product induced a T_H1 response type comparable to the vaccine expressing the native allergen, both characterized by high levels of IFN- γ and the absence of IL-5 in supernatants of splenocyte cultures. Only low levels of IL-10 could be measured (Fig 2, C). These data indicate that although B-cell epitopes have been destroyed as a result of ubiquitination, the ubi-Bet DNA vaccine still induces a remarkable immune response at the T-cell level.

The pCMV-ubi-Bet DNA vaccine protects from induction of IgE by sensitization with recombinant allergen

To assess the protective effect against a subsequent sensitization with recombinant Bet v 1, allergen-specific IgG₁, IgG_{2a}, and IgE antibodies were determined by ELISA (Fig 3, A and B), and biologically functional IgE was measured via RBL assay (Fig 3, C). Sensitization induced antibodies in all 3 groups. The response in mock immunized mice was mainly restricted to IgG₁ antibodies, whereas animals prevaccinated with pCMV-ubi-Bet or the positive control pCMV-Bet displayed a significant induction of IgG_{2a} antibodies after sensitization (Fig 3, A; $P < .005$, rank-sum test). Like the positive control group pCMV-Bet, preimmunization with pCMV-ubi-Bet fully protected from induction of functional Bet v 1-specific IgE compared with the mock vector group (Fig 3, C;

pCMV-Bet: $P < .05$; pCMV-ubi-Bet: $P = .088$; rank-sum test). A similar picture emerged from IgE measurement by ELISA (Fig 3, B; pCMV-Bet: $P < .05$, rank-sum test; pCMV-ubi-Bet: $P = .057$, rank-sum test).

Prevaccination with the ubiquitinated DNA vaccine specifically suppresses IL-5 induction and airway eosinophilia

Twenty-four hours after the last aerosol application, mice were killed, BALs were prepared, and spleens and lungs were taken for further analysis. *In vitro* restimulation of splenocytes with recombinant Bet v 1 (Fig 4, A) elicited an increased number of IFN- γ -secreting cells in both vaccination groups compared with mock immunized controls (pCMV-Bet: $P < .01$; pCMV-ubi-Bet: $P < .05$). However, probably as a result of immunostimulatory sequences containing cytidine-phosphate-guanosine (CpG) motifs, also mice that had received the empty vector showed significant IFN- γ induction compared with naive animals ($P < .05$). IL-5 secretion by splenocytes was clearly suppressed in both immunization groups compared with the mock group (pCMV-Bet: $P < .005$; pCMV-ubi-Bet: $P < .05$; rank-sum test).

The percentage of eosinophils in BALs (Fig 4, B) was significantly decreased by prevaccination with pCMV-ubi-Bet or the positive control group pCMV-Bet

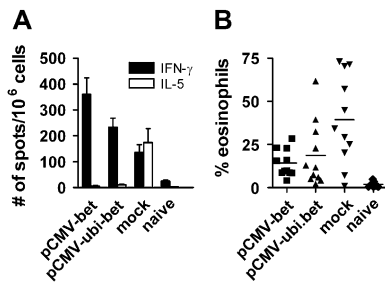


FIG 4. Measurement of key cytokines. **A**, Number of IFN- γ /IL-5-secreting splenocytes from prevaccinated (pCMV-Bet, pCMV-ubi-Bet) or mock immunized mice (*mock*) that had been sensitized and exposed to Bet v 1 aerosol. Data are shown as means $\pm$ SEMs ($n = 11$). **B**, Percentage of eosinophils in BAL fluids of the corresponding mice.

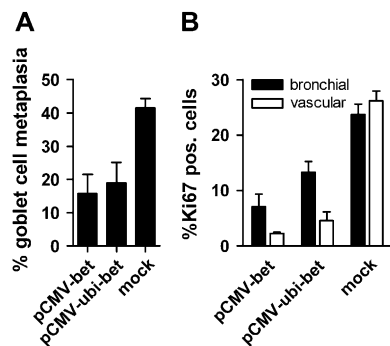


FIG 5. Analysis of mucus-producing cells and proliferating epithelium. Paraffin sections of lungs from prevaccinated (pCMV-Bet, pCMV-ubi-Bet) versus mock-immunized animals (*mock*) after sensitization and exposure to allergen were analyzed for the percentage of mucus producing goblet cells on periodic acid-Schiff stained slides (**A**), and for tissue damage in the bronchial and vascular epithelium via Ki-67 immunostaining (**B**). Data are shown as means $\pm$ SEMs ($n = 5$).

(pCMV-ubi-Bet: $P < .001$; pCMV-ubi-Bet: $P < .05$; rank-sum test) compared with the mock vector group.

DNA prevaccination with the ubiquitinated gene product reduces mucus production and epithelial damage in lung tissue

Analysis of paraffin sections of lungs revealed that DNA prevaccination with either pCMV-ubi-Bet or pCMV-Bet significantly reduced the number of mucus producing cells in the bronchus (Fig 5, A; $P < .01$) and also reduced tissue damage in the bronchial (pCMV-Bet: $P < .001$; pCMV-ubi-Bet: $P < .01$), and especially in the vascular epithelium (pCMV-Bet: $P < .001$; pCMV-ubi-Bet: $P < .001$) of the lung (Fig 5, B).

pCMV-ubi-Bet downregulates IgE levels in presensitized animals

To evaluate the therapeutic capacity of pCMV-ubi-Bet, mice with an already established T_H2-type immune response characterized by significant IgE production were treated with pCMV-ubi-Bet. The empty pCMV vector

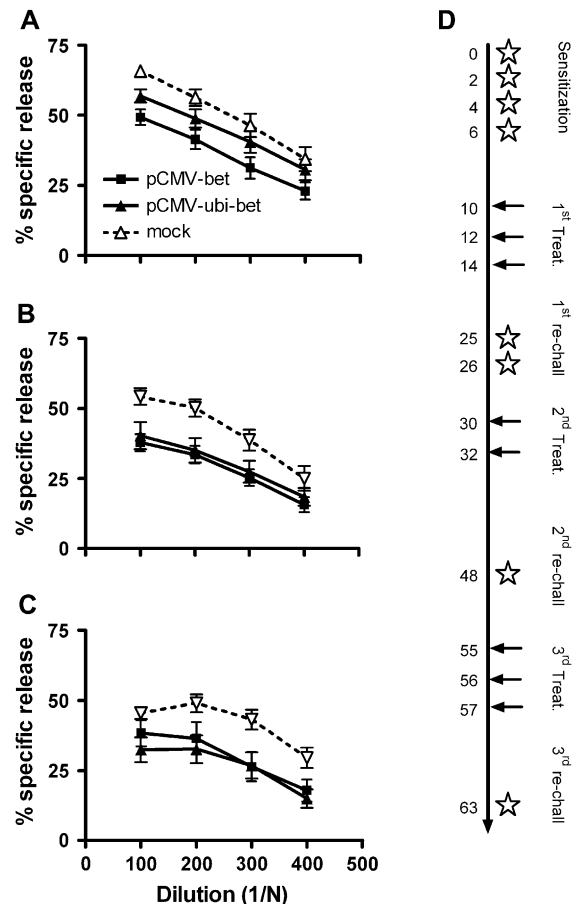


FIG 6. IgE levels in presensitized animals treated with DNA vaccines (pCMV-Bet, pCMV-ubi-Bet) or empty mock vector (*mock*). **A-C**, Specific mediator release values from RBL cells sensitized with serially diluted sera from weeks 27, 52, and 64. Data are shown as means $\pm$ SEMs of sera from individual animals ($n = 8$). **D**, Overview of the experimental schedule. Indicated time points for sensitization, treatment (*Treat.*), and re-challenge (*re-chall*) correspond with weeks.

and pCMV-Bet served as negative or positive controls, respectively. The experimental design is shown in Fig 6, D.

IgE levels were measured by RBL assay after each rechallenge (Fig 6, A: week 27; Fig 6, B: week 52; Fig 6, C: week 64). The DNA vaccine encoding ubiquitinated Bet v 1 and the conventional DNA vaccine pCMV-Bet proved to be equally efficient. Both significantly suppressed IgE levels in presensitized animals compared with mock treated animals at all time points ($P < .001$; ANOVA). Although the pCMV-Bet group showed an average IgE suppression of about 30% compared with the mock group at all time points, IgE suppression in the pCMV-ubi-Bet group increased from 12% to 37% over time.

DISCUSSION

Our current data demonstrate that covalent linkage of an allergen to a single ubiquitin molecule reduces expression

of the conformationally intact antigen by more than 90% and can thereby completely abrogate immunoglobulin production against the native protein. Although it has been previously demonstrated that ubiquitination can lead to enhanced MHC-I presentation and activation of cytotoxic T cells, we used this method for the first time to generate prophylactic as well as therapeutic allergen-specific T_H cells with a T_H1 profile in the absence of potentially anaphylactic native allergen.

This approach opens the way for routine production of DNA vaccines with minimal risk of side effects such as anaphylactic reactions or therapy-induced IgE production, the 2 major problems of SIT with allergen extracts.⁴ Moreover, because of the high purity of both the vaccine itself and the translated gene products, ubiquitinated DNA vaccines are optimal candidates for component resolved treatment concepts. DNA vaccines encoding ubiquitinated allergens also have the edge over strategies that use derivatives of recombinant allergens with reduced allergenic and anaphylactic activity, such as peptide immunotherapy,²⁶⁻²⁹ fragments,^{15,30,31} allergen oligomerization,^{30,32} and full-length recombinant modified allergens.^{14,33-35} All of these approaches are more costly and time-consuming and require evaluation of the individual allergen derivatives on a case-by-case basis.

DNA vaccination using allergen genes has been proven as a rather simple method for the development of tailor-made therapeutics and has shown promising results in a growing number of experimental studies.^{5-8,10,12} The few efforts that have been made to generate DNA vaccines encoding hypoallergenic molecules were restricted to using fragmented and mutated gene products.^{13,36} Similar to the analogous procedures with recombinant allergen derivatives, these approaches must be validated for each individual molecule and thus are not suitable for routine production. Therefore, we strived for a method to convert reliably any given allergen into a hypoallergenic form, and the well known mechanism of ubiquitination perfectly fulfilled these requirements.

Originally, forced ubiquitination of DNA vaccine-translated gene products was intended to improve the induction of cytotoxic T cells as a result of the known mechanism responsible for presentation of antigenic peptides on MHC class I molecules.^{17,20,22} It remained unclear how trafficking of peptides through the proteasome and the transporter associated with antigen processing (TAP) would lead to activation of T_H cells, because this requires presentation of antigenic peptides in the context of MHC class II molecules rather than MHC class I. However, it is known that peptides from both endogenous and exogenous sources bind to MHC class II in endolysosomal vesicles and major peptides on MHC class II also appear to be mainly derived from cellular rather than extracellular proteins.³⁷ Indeed, several studies have demonstrated involvement of proteasomal degradation in the presentation of cytosolic proteins on MHC-II.³⁸⁻⁴⁰

Our results clearly demonstrate that DNA-based vaccination with a plasmid encoding ubiquitinated Bet v 1 induces no antibodies, but antigen-specific helper T cells

of a T_H1 phenotype, which are reactivated on contact with the native allergen, leading to a T_H1-biased memory response as indicated by a strong induction of IgG_{2a} after boosting. In contrast, mock immunized mice displayed a typical T_H2-type reaction specified by the almost exclusive production of IgG₁. This was also reflected by the cytokine profile of splenocytes analyzed after sensitization with allergen, which was dominated by IFN- γ and displayed no IL-5-secreting cells after preimmunization with the pCMV-ubi-Bet DNA vaccine. Although the mock immunized control group showed significantly increased numbers of IL-5-secreting cells as expected, there were also elevated numbers of IFN- γ -secreting cells compared with naive animals, which can be attributed to unspecific stimulation via CpG motifs in the vector backbone.⁴¹

Virus-induced T_H1 reactions have been reported as a precipitating factor in asthma pathology^{42,43}; however, we have found no evidence for T_H1-mediated inflammation induced by DNA vaccination. On the contrary, DNA immunization resulted in a significant reduction of eosinophil recruitment into the lung on aerosol challenge by 64% (pCMV-Bet) and 52% (pCMV-ubi-Bet), respectively. Because eosinophilia does not necessarily correlate with lung pathology in asthma,⁴⁴ we also investigated goblet cell metaplasia and epithelial damage in the bronchial and vascular epithelium as highly relevant parameters for lung pathology. Both were significantly reduced in the ubiquitination group as well as in the positive control group (compared with the mock immunized group), suggesting a pronounced anti-inflammatory effect of both DNA vaccines on the lung tissue rather than vaccine-induced T_H1 immunopathology.

DNA vaccines have not only protective but also therapeutic potential. Especially the latter is required for the treatment of established allergic responses. Until now, only a limited number of studies could demonstrate antigen-specific therapeutic effects by DNA vaccination,^{6,8,10,11,13,45} whereas others reported inefficient therapy approaches.⁴⁶ Because of the demanding schedules for therapeutic experiments, at this time we can present only data demonstrating IgE suppression in presensitized animals. Our experiments show significant downregulation of IgE after therapy with the DNA vaccine encoding ubiquitinated Bet v 1, with a slight delay regarding establishment of maximum suppression compared with the positive control pCMV-Bet. Whether DNA vaccination can also alleviate lung pathology in mice sensitized and challenged by aerosol remains to be elucidated. Also, therapeutic schedules still have to be optimized to increase efficacy. Nevertheless, compared with classic SIT, which requires high numbers of therapeutic injections over a long period, the results from DNA-based desensitization are very promising.

Currently there is definitely an urgent need for better therapeutic approaches. However, given the fact that the prevalence of type I allergies is constantly rising and direct and indirect costs for allergic diseases and asthma have sharply increased within the past 10 years,⁴⁷ a

prophylactic vaccination against allergy will gain focus in the long run. With respect to this aim, safety optimization of vaccine formulations, as presented in our current work, will be the major issue. Taken together, the presented results demonstrate for the first time that forced ubiquitination is a versatile method to generate hypoallergenic DNA-based vaccines for prevention and treatment of allergy. Insertion of any gene of interest into a ubiquitin vector and the universal mechanism of ubiquitination enable the routine conversion of any given allergen into an effective hypoallergenic derivative and will bring DNA-based allergy vaccines one step closer to the clinic.

REFERENCES

- Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases: a WHO position paper. *J Allergy Clin Immunol* 1998;102:558-62.
- Van Ree R, Van Leeuwen WA, Dieges PH, Van Wijk RG, De Jong N, Brewczynski PZ, et al. Measurement of IgE antibodies against purified grass pollen allergens (Lol p 1, 2, 3 and 5) during immunotherapy. *Clin Exp Allergy* 1997;27:68-74.
- Ball T, Sperr WR, Valent P, Lidholm J, Spitzauer S, Ebner C, et al. Induction of antibody responses to new B cell epitopes indicates vaccination character of allergen immunotherapy. *Eur J Immunol* 1999;29:2026-36.
- Valenta R, Vrtala S, Focke-Tejkl M, Twardosz A, Swoboda I, Bugajska-Schretter A, et al. Synthetic and genetically engineered allergen derivatives for specific immunotherapy of type I allergy. *Clin Allergy Immunol* 2002;16:495-517.
- Adel-Patient K, Cremonin 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.
- 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 U S A* 1996;93:5141-5.
- 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;2:540-4.
- 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;20:1761-8.
- 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;5:387-91.
- Maecker HT, Hansen G, Walter DM, DeKruyff RH, Levy S, Umetsu DT. Vaccination with allergen-IL-18 fusion DNA protects against, and reverses established, airway hyperactivity in a murine asthma model. *J Immunol* 2001;166:959-65.
- Hartl A, Hochreiter R, Stepanoska T, Ferreira F, Thalhamer J. Characterization of the protective and therapeutic efficiency of a DNA vaccine encoding the major birch pollen allergen Bet v 1a. *Allergy* 2004;59:65-73.
- Hartl A, Kiesslich J, Weiss R, Bernhaupt A, Mostböck S, Scheiblhofer 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;103:107-13.
- Hochreiter R, Stepanoska T, Ferreira F, Valenta R, Vrtala S, Thalhamer J, et al. Prevention of allergen-specific IgE production and suppression of an established Th2-type response by immunization with DNA encoding hypoallergenic allergen derivatives of Bet v 1, the major birch-pollen allergen. *Eur J Immunol* 2003;33:1667-76.
- Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, et al. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J* 1998;12:231-42.
- Vrtala S, Akdis CA, Budak F, Akdis M, Blaser K, Kraft D, et al. T cell epitope-containing hypoallergenic recombinant fragments of the major birch pollen allergen, Bet v 1, induce blocking antibodies. *J Immunol* 2000;165:6653-9.
- Liu YC, Penninger J, Karin M. Immunity by ubiquitylation: a reversible process of modification. *Nat Rev Immunol* 2005;5:941-52.
- Rodriguez F, Zhang J, Whitton JL. DNA immunization: ubiquitination of a viral protein enhances cytotoxic T-lymphocyte induction and antiviral protection but abrogates antibody induction. *J Virol* 1997;71:8497-503.
- Delogu G, Howard A, Collins FM, Morris SL. DNA vaccination against tuberculosis: expression of a ubiquitin-conjugated tuberculosis protein enhances antimycobacterial immunity. *Infect Immun* 2000;68:3097-102.
- Zhang M, Obata C, Hisaeda H, Ishii K, Murata S, Chiba T, et al. A novel DNA vaccine based on ubiquitin-proteasome pathway targeting "self"-antigens expressed in melanoma/melanocyte. *Gene Ther* 2005;12:1049-57.
- Wu Y, Kipps TJ. Deoxyribonucleic acid vaccines encoding antigens with rapid proteasome-dependent degradation are highly efficient inducers of cytolytic T lymphocytes. *J Immunol* 1997;159:6037-43.
- Leachman SA, Shylankovich M, Slade MD, Levine D, Sundaram RK, Xiao W, et al. Ubiquitin-fused and/or multiple early genes from cottontail rabbit papillomavirus as DNA vaccines. *J Virol* 2002;76:7616-24.
- Fu TM, Guan L, Friedman A, Ulmer JB, Liu MA, Donnelly JJ. Induction of MHC class I-restricted CTL response by DNA immunization with ubiquitin-influenza virus nucleoprotein fusion antigens. *Vaccine* 1998;16:1711-7.
- Kozak M. Adherence to the first-AUG rule when a second AUG codon follows closely upon the first. *Proc Natl Acad Sci U S A* 1995;92:2662-6.
- Ecker DJ, Stadel JM, Butt TR, Marsh JA, Monia BP, Powers DA, et al. Increasing gene expression in yeast by fusion to ubiquitin. *J Biol Chem* 1989;264:7715-9.
- Hartl A, Weiss R, Hochreiter R, Scheiblhofer S, Thalhamer J. DNA vaccines for allergy treatment. *Methods* 2004;32:328-39.
- Verhoef A, Alexander C, Kay AB, Larche M. T cell epitope immunotherapy induces a CD4+ T cell population with regulatory activity. *PLoS Med* 2005;2:253-61.
- Hoyne GF, O'Hehir RE, Wraith DC, Thomas WR, Lamb JR. Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. *J Exp Med* 1993;178:1783-8.
- Muller U, Akdis CA, Fricker M, Akdis M, Blesken T, Bettens F, et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. *J Allergy Clin Immunol* 1998;101:747-54.
- Janssen EM, van Oosterhout AJ, van Rensen AJ, van Eden W, Nijkamp FP, Wauben MH. Modulation of Th2 responses by peptide analogues in a murine model of allergic asthma: amelioration or deterioration of the disease process depends on the Th1 or Th2 skewing characteristics of the therapeutic peptide. *J Immunol* 2000;164:580-8.
- van Hage-Hamsten M, Kronqvist M, Zetterstrom O, Johansson E, Niederberger V, Vrtala S, et al. Skin test evaluation of genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1: results obtained with a mix of two recombinant Bet v 1 fragments and recombinant Bet v 1 trimer in a Swedish population before the birch pollen season. *J Allergy Clin Immunol* 1999;104:969-77.
- Wiedermann U, Herz U, Vrtala S, Neuhaus-Steinmetz U, Renz H, Ebner C, et al. Mucosal tolerance induction with hypoallergenic molecules in a murine model of allergic asthma. *Int Arch Allergy Immunol* 2001;124:391-4.
- Vrtala S, Hirtenlehner K, Susani M, Akdis M, Kussebi F, Akdis CA, et al. Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen Bet v 1. *FASEB J* 2001;15:2045-7.
- Akdis CA, Blesken T, Wymann D, Akdis M, Blaser K. Differential regulation of human T cell cytokine patterns and IgE and IgG4 responses by conformational antigen variants. *Eur J Immunol* 1998;28:914-25.
- Vailes LD, Li Y, Bao Y, DeGroot H, Aalberse RC, Chapman MD. Fine specificity of B-cell epitopes on *Felis domesticus* allergen I (Fel d I): effect of reduction and alkylation or deglycosylation on Fel d I structure and antibody binding. *J Allergy Clin Immunol* 1994;93:22-33.

35. Smith AM, Chapman MD, Taketomi EA, Platts-Mills TA, Sung SS. Recombinant allergens for immunotherapy: a Der p 2 variant with reduced IgE reactivity retains T-cell epitopes. *J Allergy Clin Immunol* 1998;101:423-5.
36. Hartl A, Kiesslich J, Weiss R, Bernhaupt A, Mostböck S, Scheibelhofer 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.
37. Rudensky A, Preston-Hurlburt P, Hong SC, Barlow A, Janeway CA Jr. Sequence analysis of peptides bound to MHC class II molecules. *Nature* 1991;353:622-7.
38. Mukherjee P, Dani A, Bhatia S, Singh N, Rudensky AY, George A, et al. Efficient presentation of both cytosolic and endogenous transmembrane protein antigens on MHC class II is dependent on cytoplasmic proteolysis. *J Immunol* 2001;167:2632-41.
39. Bonifaz LC, Arzate S, Moreno J. Endogenous and exogenous forms of the same antigen are processed from different pools to bind MHC class II molecules in endocytic compartments. *Eur J Immunol* 1999;29:119-31.
40. Lich JD, Elliott JF, Blum JS. Cytoplasmic processing is a prerequisite for presentation of an endogenous antigen by major histocompatibility complex class II proteins. *J Exp Med* 2000;191:1513-24.
41. Jilek S, Barbey C, Spertini F, Corthesy B. Antigen-independent suppression of the allergic immune response to bee venom phospholipase A(2) by DNA vaccination in CBA/J mice. *J Immunol* 2001;166:3612-21.
42. Holtzman MJ, Agapov E, Kim E, Kim JI, Morton JD. Developing the epithelial, viral, and allergic paradigm for asthma: Giles F. Filley lecture. *Chest* 2003;123:377S-84S.
43. O'Sullivan SM. Asthma death, CD8+ T cells, and viruses. *Proc Am Thorac Soc* 2005;2:162-5.
44. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000;356:2144-8.
45. Jarman ER, Lamb JR. Reversal of established CD4+ type 2 T helper-mediated allergic airway inflammation and eosinophilia by therapeutic treatment with DNA vaccines limits progression towards chronic inflammation and remodelling. *Immunology* 2004;112:631-42.
46. Sudowe S, Montermann E, Steitz J, Tuting T, Knop J, Reske-Kunz AB. Efficacy of recombinant adenovirus as vector for allergen gene therapy in a mouse model of type I allergy. *Gene Ther* 2002;9:147-56.
47. Bousquet J, Ansotegui IJ, van Ree R, Burney PG, Zuberbier T, van Cauwenberge P. European Union meets the challenge of the growing importance of allergy and asthma in Europe. *Allergy* 2004;59:1-4.