

Original article

Characterization of the protective and therapeutic efficiency of a DNA vaccine encoding the major birch pollen allergen Bet v 1a

Background: An estimated 100 million individuals suffer from birch pollen allergy. More than 95% of birch pollen-allergic subjects react with the major birch pollen allergen Bet v 1a, and almost 60% of them are sensitized exclusively to this allergen.

Objective: DNA immunization using the Bet v 1a gene was evaluated with respect to its prophylactic and therapeutic efficacy.

Methods: A DNA vaccine containing the entire Bet v 1a cDNA under the control of a CMV-promoter was constructed. In order to estimate the protective efficiency, animals received three injections of this vaccine prior to sensitization with recombinant Bet v 1a. Vice versa, in a therapeutic approach, sensitization was followed by treatment with the DNA vaccine.

Results: The Bet v 1a DNA vaccine induced strong Bet v 1-specific antibody responses with a Th1-biased response type. Animals which received the DNA vaccine were protected against a following allergic sensitization with Bet v 1a. The protective effect was characterized by suppression of Bet v 1-specific immunoglobulin (Ig)E production, lack of basophil activation and enhanced interferon (IFN)- γ expression. In a therapeutic situation, treatment of sensitized animals with DNA vaccines decreased IgE production, IgE-mediated basophil release and drastically reduced anaphylactic activity as measured by passive cutaneous anaphylaxis assays. Concerning the cellular immune response, DNA immunization induced a sustaining and dominant shift from a Th2 type response towards a balanced Th1/Th2 type response as indicated by increased IFN- γ but unchanged IL-5 levels in lymphoproliferation assays.

Conclusion: The results demonstrate the allergen-specific protective and therapeutic efficacy of a DNA vaccine encoding the clinically highly relevant allergen Bet v 1a indicating the suitability of this concept for the treatment of allergic diseases.

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Type I allergic symptoms are caused by mediators released from effector cells (mast cells, basophils) after cross linking of immunoglobulin (Ig)E bound to Fc ϵ RI receptor by the allergen (1, 2). Sensitization, the abnormal switch to IgE production against ubiquitous proteins, takes place early in childhood, is triggered by interleukin (IL)-4 producing Th2 type cells and leads to the establishment of specific IgE antibody responses which are boosted by repeated allergen contact (3–6).

Recent approaches such as treatment with IgE-blocking antibodies (7) or inhibition of IL-4 binding (8) have addressed these aspects. Nevertheless, the application of specific immunotherapy (SIT) still represents the only curative approach towards therapy of IgE-mediated allergies (9, 10). However, SIT is also an inconvenient procedure with a high rate of undesirable side effects and the requirement of intensive medical supervision (10–12).

Moreover, despite the long history of SIT, its mechanisms are not fully understood (13).

In the past years, DNA immunization was demonstrated as a powerful alternative to protein based vaccination (14). It has been shown that intramuscular or intradermal injection of plasmid DNA encoding allergens can induce immune responses with a Th1 bias and promote the formation of interferon (IFN)- γ producing CD4+ T-cells (15–22).

In the present study we investigated both the protective and therapeutic potential of a DNA vaccine encoding Bet v 1a. We could demonstrate that the DNA vaccine induced a Th1-biased immune response type against Bet v 1a which both, protected from IgE antibody production and IgE-mediated cell release after sensitization, as well as converted an established Th2 type response. The findings are discussed regarding the use of DNA vaccines for specific prophylaxis and therapy of allergic diseases.

Material and methods

Experimental design

To evaluate the protective effect of DNA vaccination against the induction of an allergic Th2-immune response, groups of nine female BALB/c mice received three intradermal injections of plasmid DNA coding for the major birch pollen allergen Bet v 1a (pCMV-Betv1) in a 1-week interval. At days 57 and 64 after the first injection, five animals of each group were sensitized with recombinant Bet v 1a (rBetv1). The remaining four animals served as nonsensitized control. In addition a group of six untreated animals was sensitized with rBetv1.

In order to address the question whether the effects of DNA preimmunization were antigen-specific or because of the adjuvant effect of Th1-inducing CpG motifs present in the plasmid DNA, another group of six animals was preimmunized with a plasmid DNA-construct encoding the timothy grass pollen allergen Phl p 5 (pCMV-Phlp5) using the same immunization schedule.

To study the therapeutical efficacy of the Bet v 1 DNA-vaccine, groups of six female BALB/c mice received two subcutaneous injections (day 0 and 7) of rBetv1. In that manner sensitized mice were treated with plasmid DNA coding for Bet v 1a (pCMV-Betv1) or mock vector at day 63 and 76. Another group of six animals was injected with recombinant allergen according to the same immunization-schedule but received no plasmid DNA.

Immune responses were analyzed by enzyme-linked immunosorbent assay (ELISA), lymphoproliferation and cytokine (IFN- γ and IL-5) measurement in culture supernatants. Passive cutaneous anaphylaxis assay (PCA) and rat basophil cell release assay served as functional read-out. The whole experiment was performed twice.

Construction of pCMV-Bet DNA immunization vector

The expression plasmid pCMV-Betv1 was constructed as previously described (18). The plasmids were propagated in *Escherichia coli* XL1-blue (Stratagene, La Jolla, CA). Large scale purification of the expression vectors were conducted with Endo Free Plasmid Giga kits (Qiagen, Hilden, Germany) according to the manufacturers 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).

Immunization and sera collection

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 Austrian guidelines for animal care.

For sensitization, mice were injected with 5 μ g of rBetv1 together with 100 μ l Al(OH)₃ (2 mg/ml; Serva, Heidelberg, Germany) in a total volume of 150 μ l sterile phosphate-buffered saline (PBS).

The DNA immunization was given intradermally into the shaved back with 100 μ g of plasmid DNA in a volume of 200 μ l sterile PBS distributed in six spots. Mice were bled through the tail vein; sera were stored at 4°C for later analysis.

ELISA for measurement of Ab titers, isotypes and avidity

Black 96-well high-bind immunoplates (Greiner, Kremsmünster, Austria) were coated by overnight incubation at 4°C with 100 ng rBetv1/well in PBS and ELISA was performed as described (15, 23).

Proliferation assay and cytokine detection

Preparation of splenocytes, proliferation assays and determination of cytokines from supernatants of proliferation cultures were performed as described (15).

PCA reactions

Anaphylactic activity of sera obtained from immunized mice was determined by passive cutaneous anaphylaxis reactions in naive Balb/c mice as described (24). Briefly, pooled sera were diluted 1 : 10 in sterile PBS and 30 μ l of diluted serum were injected intradermally on the shaved back. After 2 h the animals were challenged i.v. with 100 μ l of 0.5% Evan's blue solution containing 250 μ g of birch pollen protein extract. All tests were made in triplicate; samples of DNA-treated and untreated mice that were to be compared were injected into the same animal.

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 murine sera at a final dilution of 1 : 30 for 2 h. To remove unbound antibodies, the cell layer was washed two 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 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 0.1% bovine serum albumin, pH 7.2). Cross-linking of the Fc ϵ R-bound IgE and subsequent degranulation of RBL cells was induced by adding 100 μ l rBetv1 ($c = 0.3$ μ g/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 μ M 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide (Sigma-Aldrich, Vienna, Austria) in citrate buffer (0.1 M, pH 4.5) for 1 h at 37°C. The reaction was stopped by addition of 100 μ l glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and fluorescence was measured at λ_{ex} : 360/ λ_{em} :465 nm using a fluorescence microplate reader (Spectrafluor, Tecan, Salzburg, Austria). Results are reported as percentage of total β -hexosaminidase released after addition of 10% Triton X-100.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical significance was assessed by the nonparametric Mann-Whitney rank sum test.

Results

DNA immunization modulates the humoral response against Bet v 1a with, both, a protective as well as a therapeutic experimental approach

The primary response of animals immunized with rBetv1 protein (Fig. 1A) as well of those genetically immunized with pCMV-Betv1 (Fig. 1B) revealed a strong humoral immune reaction. The subclass response after protein immunization was restricted mainly to IgG1, whereas DNA immunization induced the expression of high levels of allergen-specific IgG1 and IgG2a antibodies. To

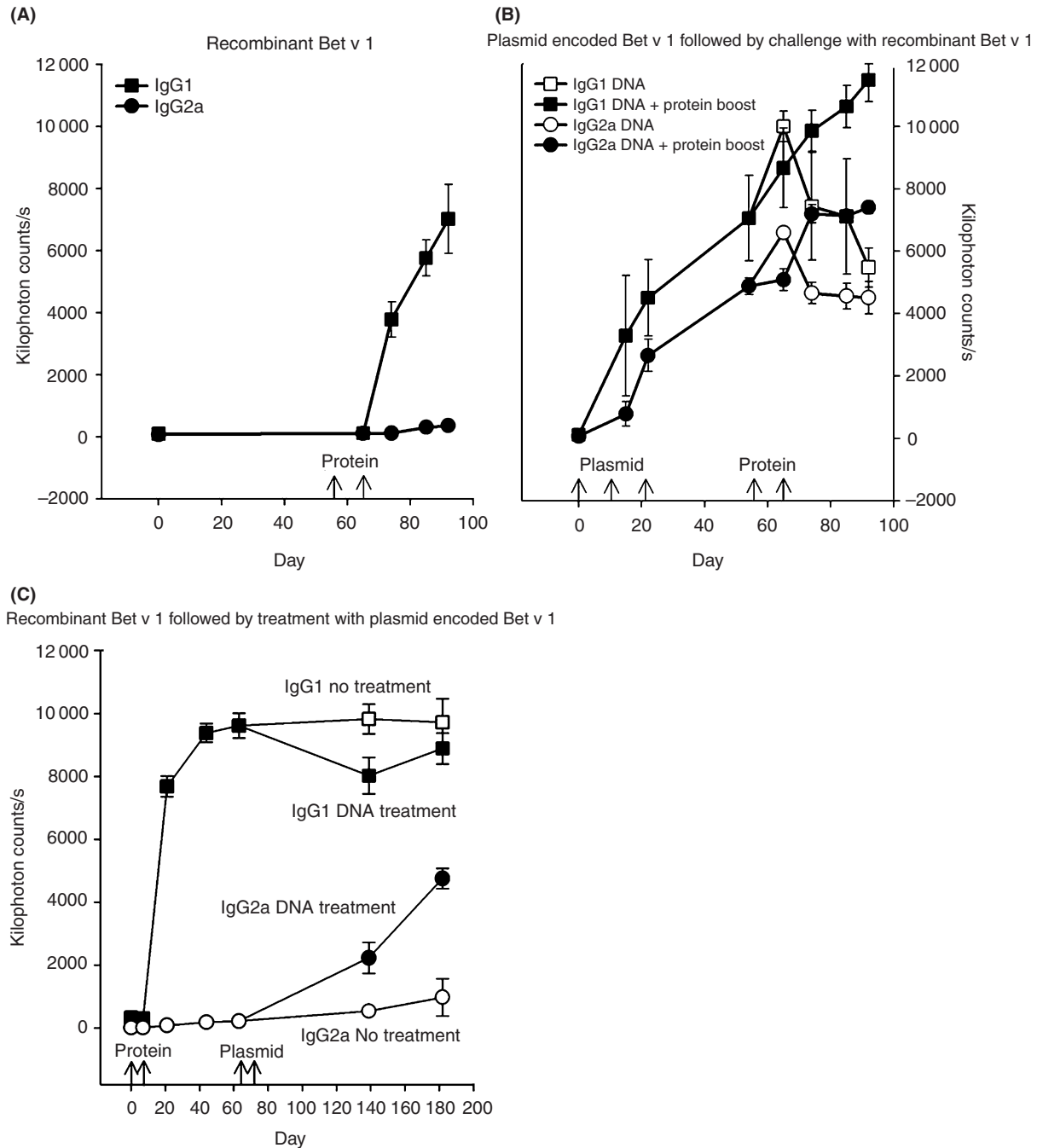


Figure 1. Bet v1-specific immunoglobulin (Ig)G1 and IgG2a immune responses. To study the protective effect, BALB/c mice ($n = 9$) were immunized i.d. with pCMV-Betv1, three times in a weekly interval. On days 57 and 64, animals were sensitized s.c. with recombinant Bet v 1a (rBetv1) ($n = 5$) or left untreated ($n = 4$) (B). To evaluate the therapeutical efficacy, groups of six female BALB/c mice were sensitized with two subcutaneous injections (day 0 and 7) of rBetv1 and treated with pCMV-Betv1 or mock vector at day 63 and 76 (C). A control group ($n = 6$) received two injections of recombinant Bet v 1 (A). Bet v1-specific antibody responses were measured by enzyme-linked immunosorbent assay and shown as kilophoton counts/s on the y-axis. Immunizations are indicated by arrows, data are expressed as mean $\pm$ SEM.

investigate whether the Th1-type stimulus induced by intradermal DNA immunization has any effect on the subsequent response induced by protein immunization (protective approach), mice were sensitized two times with

5 μ g rBetv1 adsorbed to $\text{Al}(\text{OH})_3$ 5 weeks after the last DNA immunization. The protein boost induced a further increase of IgG1 and IgG2a antibodies (Fig. 1B). The antibody levels in animals without protein sensitization

reached the highest values at 60 days after the first injection and then declined (Fig. 1B). The influence of the DNA-preimmunization can also be seen by comparing Fig. 1A and B, which demonstrates that DNA immunization with Bet v 1a modulates the subsequent protein response by inducing elevated levels of allergen-specific IgG2a antibodies ($P < 0.001$ compared with protein immunization). In contrast, in the same experiment, protein immunization of a group of animals without DNA preimmunization primarily induced IgG1 antibodies and only very low levels of IgG2a antibodies (Fig. 1A).

Preimmunization with a DNA vaccine encoding the Bet v 1 unrelated allergen Phl p 5 (serving as control vector) induced strong induction of IgG1 and IgG2a antibodies against Phl p 5 but revealed no alteration of the Bet v 1-specific serological Th-type response after sensitization with rBetv1 (data not shown).

Using a therapeutic approach, animals were sensitized by two injections of 5 μ g rBetv1 adsorbed to Al(OH)₃, and 8 weeks later, treated with two injections of pCMV-Betv1 in a weekly interval (Fig. 1C). The DNA immunization induced a booster effect for IgG2a and a transient suppression for IgG1 antibodies indicating a Th1-directed modulatory effect of the DNA vaccine on the established Th2-type reaction (Fig. 1C).

DNA immunization specifically inhibits the induction of Bet v 1-specific IgE responses and suppresses IgE production of an established Th2 type response

Figure 2A shows the levels of specific anti-Bet v 1a IgE antibodies in the serum 4 weeks after sensitization with protein. Genetic preimmunization with pCMV-Betv1 was able to prevent the induction of IgE antibodies induced by protein sensitization ($P = 0.01$).

In contrast, preimmunization of animals with the irrelevant pCMV-Phlp5 and subsequent sensitization with rBetv1 did not prevent the induction of anti-Bet v 1a IgE antibodies (Fig. 2A).

Furthermore, DNA immunization elicited the IgE-suppressing effects also in the therapeutic situation. For balancing an already established Th2 reaction two injections of pCMV-Betv1 were sufficient to reduce the level of IgE by 70% 10 weeks after the second plasmid injection (Fig. 2B). Treatment of the animals with a vector lacking the insert did not reduce the IgE levels thus indicating the antigen-specific nature of the anti-allergic effect of the Bet v 1 DNA vaccine (Fig. 2B).

DNA immunization and protein immunization induce different antiserum avidities

Antibody affinity plays an important role in binding and crosslinking of antigen on the surface of mast cells and basophils and thus also influences the capacity of sera to trigger cell release and induce anaphylactic effects. Therefore we measured the serum avidity by disrupting

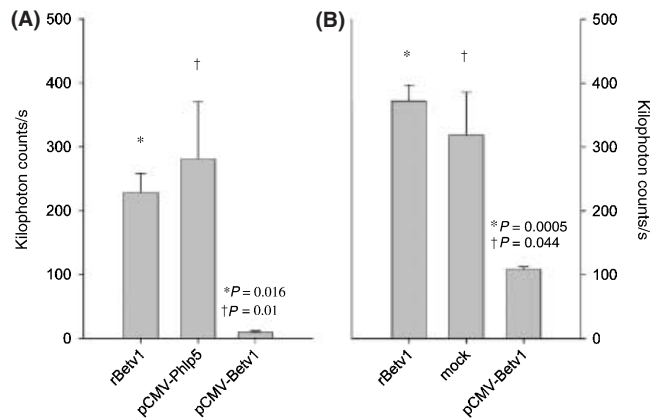


Figure 2. Levels of Bet v 1a-specific immunoglobulin (IgE). Panel A: IgE responses of mice pretreated with pCMV-Betv1 or pCMV-Phlp5 followed by sensitization with recombinant Bet v 1a (rBetv1). Blood was taken 4 weeks after the last sensitization (day 92). rBetv1, sensitization with recombinant Bet v 1a without DNA preimmunization. Panel B: IgE responses of mice sensitized with recombinant Bet v 1a and treated with pCMV-Betv1 or mock vector. Blood was taken 10 weeks after the second plasmid-DNA injection (day 139). rBetv1, sensitization without DNA-treatment.

antigen-antibody interactions with increasing concentrations of the chaotropic agent sodium thiocyanate (NaSCN).

The avidity of sera from experimental and control groups were determined at day 92 in the protection approach (Fig. 3A) and at day 139 in the therapy approach (Fig. 3B). Comparing the avidity of control sera from animals which received only DNA immunization or protein immunization revealed that protein immunization (Effective Dose 50 = 1.6 M NaSCN) induced higher avidity values than DNA immunization (Effective Dose 50 = 1.0 M NaSCN). Protein-boosting of animals preimmunized with a DNA vaccine increased the avidity of all detectable antibody subclasses. In contrast, DNA immunization reduced the protein-induced avidity of the detectable antibody subclasses. The latter indicates that the DNA vaccine obviously modulated the pattern of existing B-cells and new B-cells with different epitope specificities and/or affinities were recruited.

DNA immunization prevents and suppresses Bet v 1-induced immediate reactions

In order to determine the extent to which DNA immunization can prevent immediate type reactions, an effector cell test was used. The measurement of allergen-specific degranulation of RBL-2H3 cells passively sensitized with murine sera was performed by the detection of β -hexosaminidase release after cross-linking of receptor bound antibodies with rBetv1 (Fig. 4). Sera from Bet v 1a

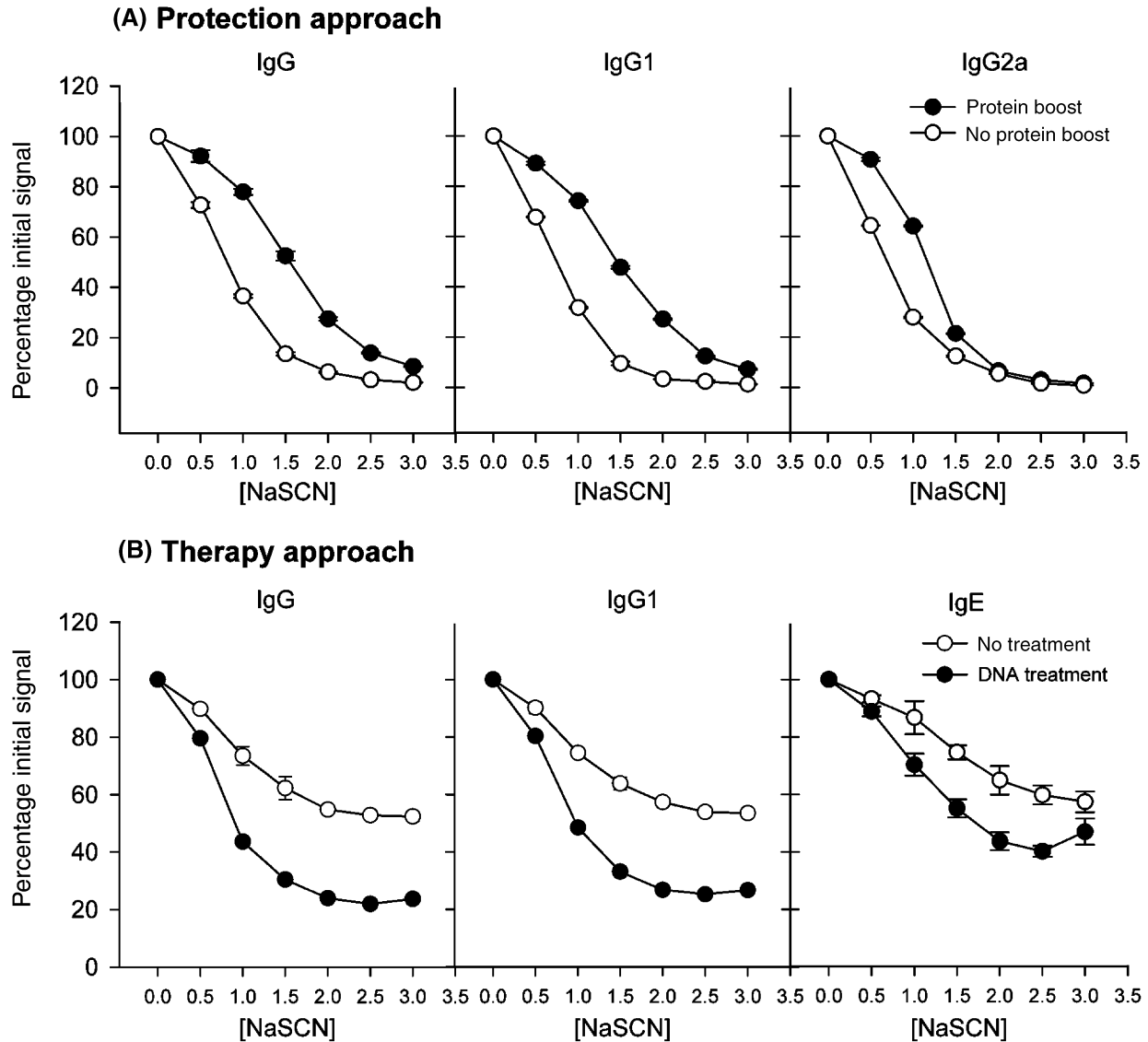


Figure 3. Avidity of Bet v 1a-specific antibodies. In the protection approach avidity was determined at day 92 (A), in the therapy approach at day 139 (B). The antigen-antibody complexes were treated with increasing concentrations of NaSCN. The amount of bound antibodies remaining after NaSCN was determined as a percentage of bound antibodies in the absence of chaotropic reagent and plotted against molar concentrations of NaSCN.

protein-sensitized mice contained Bet v 1-specific IgE (Fig. 2A) and thus induced Bet v 1-specific basophil degranulation (white bars, Fig. 4A). By contrast, sera from mice pretreated with Bet v 1a-specific DNA immunization did not induce basophil degranulation regardless of a subsequent Bet v 1a protein injection was given (dark gray bars) or not (light gray bars, Fig. 4A).

Sera genetically pre-immunized with Phl p 5 plasmid DNA displayed no anaphylactic activity when cross-linked with rBetv1 (light gray bars, Fig. 4A). However, sera from animals genetically preimmunized with Phl p 5 plasmid DNA and boosted with rBetv1 contained Bet v

1-specific IgE and induced basophil release (dark gray bars, Fig. 4A), indicating the antigen-specific nature of the protective effect.

In a therapeutic situation, DNA vaccination was able to balance the effect of an ongoing allergic response in an antigen-specific manner, too. After treatment with pCMV-Betv1 the level of the allergen-specific degranulation was reduced to 55% (Fig. 4b). In contrast, treatment with a vector lacking the insert revealed no significant effect. Furthermore, a PCA showed that the Th2-converting effect of the Bet v 1 DNA vaccine continuously developed over a period of weeks and reached its maximum efficiency at day 182 (Fig. 4C).

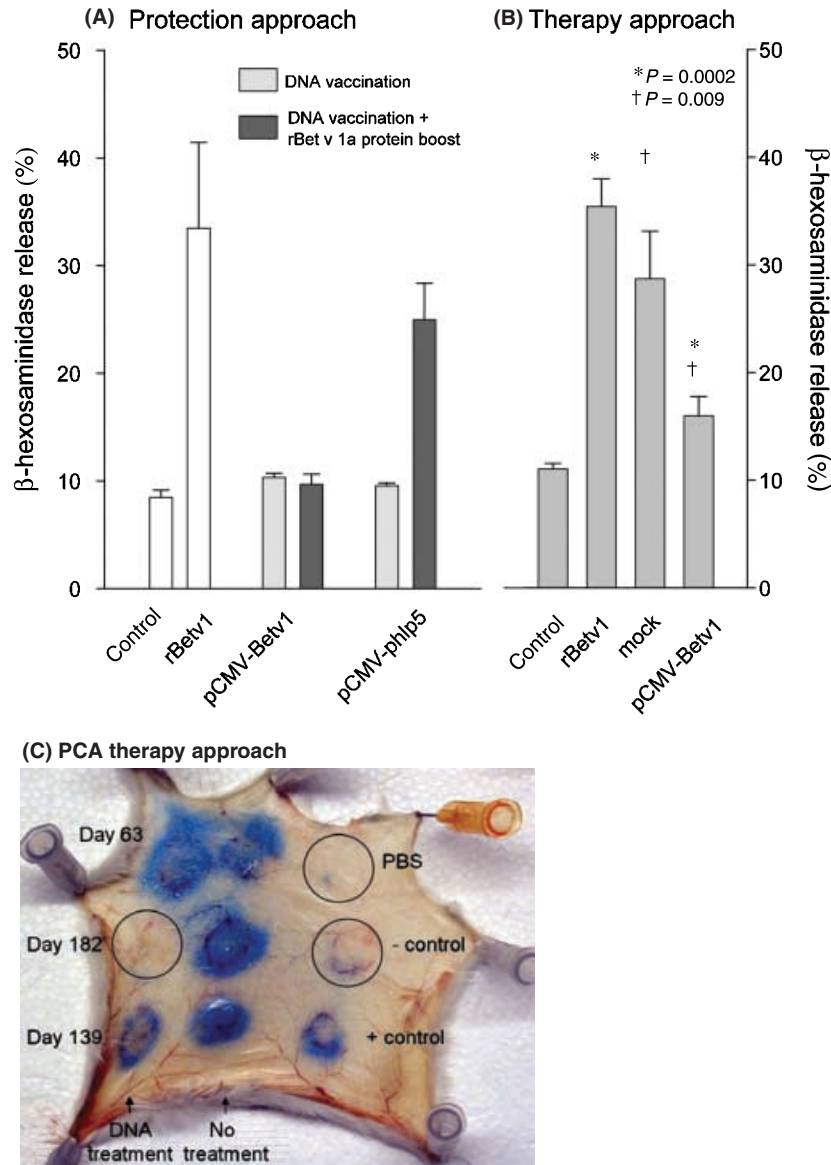


Figure 4. Rat basophil leukemia cell release assay and passive cutaneous anaphylaxis (PCA) assay. Analogous to Fig. 2, sera from day 92 of the protection approach (A) and from day 139 of the therapy approach (B) were tested for their anaphylactic activity by measuring the release of β -hexosaminidase. In a PCA-assay (C), pooled sera ($n = 6$, DNA treatment) from day 63 (1 day before DNA-treatment), day 139 and day 182 were compared with sera harvested at the same time from the untreated control group ($n = 6$, no treatment). Phosphate-buffered saline, sera from na mice (–control) and from mice immunized with recombinant Bet v 1 (+ control) served as controls.

Protective and therapeutic DNA immunization recruits antigen-specific Th1 cells

To investigate whether a long-lasting and allergen-specific Th1 memory was induced by DNA preimmunization, *in vitro* stimulation of spleen cells from animals of all different groups was performed with rBetv1 (Fig. 5A). The antigen-specificity of each group was proven by stimulation with ovalbumin, which gave values comparable to the nonimmunized control group.

In the protective approach, lymphoproliferation assays performed 230 days after the last injection of Plasmid DNA, still revealed a significantly elevated stimulation index (Fig. 5A). Protein boosting of DNA-preimmunized animals did not enhance the proliferative activity of spleen cells and the proliferative response after protein immunization with rBetv1 alone was comparable to that of the other immunization groups (Fig. 5A).

The analysis of the culture supernatants revealed a significantly higher expression of IFN- γ in both groups preimmunized with plasmid DNA ($P < 0.01$) compared with immunization with recombinant protein alone. Protein boosting after DNA immunization did not significantly increase the IFN- γ levels (Fig. 5B).

In the therapeutic approach lymphoproliferation assays were performed 110 days after the last treatment with the DNA vaccine. The proliferative response of animals that received two injections with rBetv1 protein was not influenced by two further immunizations with plasmid DNA (Fig. 5C). The response was proven to be antigen-specific as indicated by the stimulation values obtained with ovalbumin. However, DNA immunization obviously induced the recruitment of Th1 memory cells which produced IFN- γ after stimulation with the recombinant allergen ($P = 0.01$; Fig. 5D) whereas the determination of IL-5 revealed no statistically significant differences between both groups (Fig. 5E).

Discussion

Our data confirm recent publications demonstrating that DNA immunization offers a feasible alternative for the treatment of Type-1 allergic diseases in, both, a protective as well as a therapeutic way. The Th1-biased 'default response' after intradermal or intramuscular genetic immunization balanced Th2-type response conditions with model molecules (16, 25) and clinically relevant allergens (19–22, 26, 27).

In the present study this principle was demonstrated with a DNA vaccine containing the entire coding region of the major allergen of birch pollen, Bet v 1a, representing one of the most frequent environmental allergens. Preimmunization with the DNA vaccine induced high levels of IgG1 and nearly equal amounts of IgG2a antibody titers indicating a substantial Th1-type component of the response. This reaction profile did not change after a sensitization with recombinant allergen. Furthermore, DNA vaccination prevented IgE production by protein sensitization and induced functional protection against an allergic immune reaction as indicated by the inhibition of IgE-mediated basophil cell release.

A therapeutic approach with DNA immunization modulated an ongoing allergic response type including suppressing of IgE expression and IgE-mediated cell release. In addition, the therapeutic efficacy of DNA vaccines was clearly demonstrated by the *in vivo* inhibition of passive cutaneous anaphylaxis.

Both, the protective as well as the therapeutic approach, are highly antigen-specific as indicated by the control experiments with a mock vector or a vector expressing an irrelevant allergen.

At present we neither fully understand the mechanisms underlying conventional SIT nor that underlying

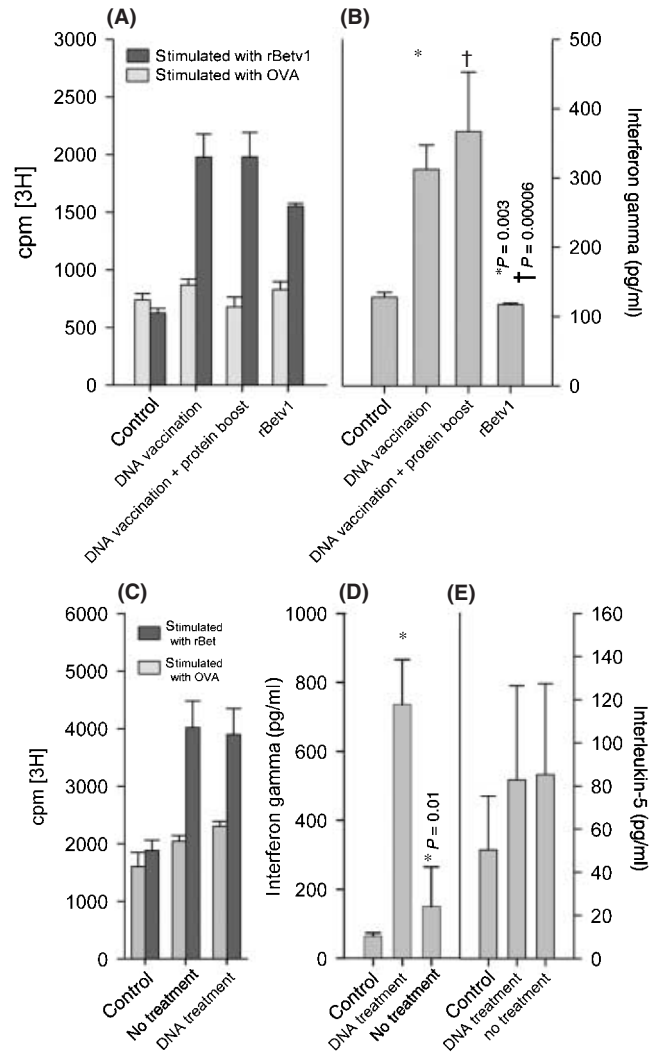


Figure 5. Proliferative response and secretion of cytokines in supernatants of stimulated spleen cells. In the protection approach, spleen cells of mice ($n = 3$) were harvested at day 230 and cultured in the presence of 20 $\mu\text{g/ml}$ rBetv1 or OVA (A). Interferon (IFN)- γ levels of rBetv1 stimulated splenocytes were measured by enzyme-linked immunosorbent assay in pg/ml (B). In the therapy approach, spleen cells of mice ($n = 3$) were harvested at day 182 and stimulated with rBetv1 or OVA (C). The Levels of IFN- γ (D) and interleukin-5 (E) in the proliferation supernatants were determined (D). Data represent mean $\pm$ SEM.

DNA-based desensitization. However, in contrast to the controversially discussed immunological mechanisms of SIT (28) the rationale for DNA immunization is clear and strictly addresses the balance of the Th1/Th2 type reaction.

The essential anti-allergic mechanisms induced by intradermal DNA immunization involve the recruitment of antigen-specific Th1 cells and the creation of a Th1 cytokine milieu. This is reflected by the data of, both, the protective as well as the therapeutic approach in this study, too.

As a consequence of sufficient antigen-specific Th1 memory cells any further presentation of the homologous antigen to these cells will force their activation into a Th1-biased direction and will increase the Th1 components as was shown by the elevation of IgG2a antibodies and IFN- γ whenever protein immunization was combined in a protective or therapeutic approach with DNA immunization.

In our experimental design, the Alum-effect (29) resembles the continuous exposure during the allergen season. By this way allergens could be the source for recruitment of Th1 memory cells and perhaps creating a sustained antigen-specific Th1 cytokine milieu as has been demonstrated by the lymphoproliferation assays and cytokine analysis.

With respect to their Bet v 1a T cell epitope-specificity, Th1 cells recruited by DNA immunization may also interact with B-cells, which have recognized, processed and presented allergen during sensitization. Therefore, the distribution of Th1 and Th2 cells after DNA immunization will determine the milieu and response type after contact with the allergen. The increase of IgG2a antibodies in the groups vaccinated with plasmid DNA and boosted with protein could be interpreted in the way that DNA immunization had created sufficient Bet v 1a-specific Th1 cells which were able to modulate the subsequent immune reaction against the allergen. On the contrary, using the therapeutic approach, the DNA vaccine obviously led to the recruitment and expansion of new Th1 cells which were able to balance the ongoing Th2 type response in an antigen-specific manner. The decrease of antibody avidity as a consequence of DNA

immunization may reflect the influence of newly recruited Th1 cells on B cell activation and maturation.

With respect to the Th1-biased immune response after intradermal DNA immunization, a frequent concern is the loss of Th2 type immunocompetence. However, as can be seen in the case of the Bet v 1 DNA vaccine and in numerous other studies dealing with genetic immunization, not only IgG2a but also high titers of IgG1 are induced by DNA vaccines pointing to activation of Th2 cells, too, and the maintenance of IL-4 pathways (30–32), which are necessary for a number of anti-parasitic immune reactions. Furthermore, the parallel existence of antigen-specific Th1 and Th2 type responses in one organism, as demonstrated in the control groups with a Phl p 5-specific Th1 type response after DNA immunization and a Bet v 1a-specific Th2 type response induced by sensitization with recombinant allergen argues against a simplistic interpretation of immunological processes.

The present study, based on the highly relevant allergen Bet v 1a, clearly demonstrates that DNA vaccines, beyond their protective properties, also display a therapeutic efficacy suggesting DNA vaccines as a safe and effective therapy for allergic diseases.

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