

Isoforms of the Major Allergen of Birch Pollen Induce Different Immune Responses after Genetic Immunization

Arnulf Hartl^a Jan Kiesslich^a Richard Weiss^a Andrea Bernhaupt^a
Sven Mostböck^a Sandra Scheiblhofer^a Hannes Flöckner^a Manfred Sippl^a
Christof Ebner^b Fátima Ferreira^c Josef Thalhamer^a

^aInstitute of Chemistry and Biochemistry, University of Salzburg; ^bInstitute of General and Experimental Pathology, University of Vienna, and ^cInstitute of Genetics and General Biology, University of Salzburg, Austria

Key Words

Bet v 1 · DNA immunization · DNA immunotherapeutics · Birch pollen allergen · Immunotherapy · BALB/c

Abstract

Background: Recent publications indicate that immunization with plasmid DNA encoding allergens might represent a potential approach in allergen-specific immunotherapy. **Objective:** In the present study we have compared the immune responses induced by plasmid DNA encoding for two isoforms of Bet v 1, the major allergen of birch pollen. **Methods:** BALB/c mice were injected intradermally with plasmid DNA encoding for the genes of Bet v 1a (pCMV-Beta) and Bet v 1d (pCMV-Betd). In addition, the effect of immunostimulatory DNA sequences was investigated by appending and/or coinjecting CpG motifs. Antibody responses and IFN- γ and IL-4 levels were measured by ELISA. Allergen-specific proliferation was determined by incorporation of [³H]-thymidine. **Results:** The two isoforms induced a similar humoral response. The lack of any IgE production and the ratio of IgG1 to IgG2a clearly indicated a Th-1-type response. The antisera against both isoforms were highly cross-reactive, which was supported by the ener-

gy plot indicating similar folding of the two protein isoforms. However, determination of IFN- γ and IL-4 in the serum elicited a strikingly different cytokine profile during the course of the immune response. In contrast to pCMV-Beta, pCMV-Betd caused no significant allergen-specific proliferation and induced only marginal levels of the key cytokines. **Conclusions:** Based on the assumption that the induction of a strong Th-1 type response is a prerequisite for successful treatment of allergy, our results favor the use of isoform Bet v 1a in combination with CpG motifs for a novel type of allergen immunotherapy based on plasmid DNA immunization. Additionally, the data also confirm the assumption that the antigen itself can have a marked influence on the immune response after genetic immunization.

Introduction

Allergic symptoms caused by type I allergies, like rhinitis, bronchial asthma and conjunctivitis, are widespread among the human population of industrialized countries. A sustained production of IgE molecules against common environmental antigens is one of the characteristic events in

allergic disease [1]. Cytokines, like IL-4 and IFN- γ are assumed to determine the course and severity of the allergic phenomenon [2–5]. Additional regulatory influences may also be exerted not only by CD4⁺ T cells but also by CD8⁺ T cells [6–8].

With genetic immunization, a new and powerful method for vaccine research and development arose in the last years [9]. Recent publications also have demonstrated a potential role of this immunization technique for allergy treatment [10–12]. Meanwhile, there is a broad consensus with respect to the mechanisms of genetic immunization and the basic events after injection of plasmid DNA [9, 13]. Both the delivery of DNA to tissue with a gene gun and intramuscular or intradermal injection of a saline-DNA solution proved to induce antigen-specific humoral and cellular responses [14–17]. In contrast to immunization with proteins, which are taken up by antigen-presenting cells (APCs) and consequently are presented via MHC-II molecules, plasmid DNA must first transfect cells to be translated into proteins. The predominant pathway therefore goes via MHC-I molecules, thus leading to the activation of a cellular immune response. However, the separation of both aspects of the immune system in genetic immunization is not that strict because translated proteins also seem to be available for APCs to induce humoral responses [18–20].

A special feature of this method, and the major difference between conventional immunization with protein antigens and plasmid DNA, is the induction of an INF- γ -mediated Th-1 type response. Certain DNA motifs, consisting of an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines, offer an explanation for these results. These immunostimulatory CpG motifs are 20 times as common in bacterial DNA than in mammalian DNA, and may serve as a 'danger signal' for the mammalian immune system. They were shown to stimulate the innate immune system to produce a number of Th-1-inducing cytokines (including IL-12, IL-18, IFN- α , β and TNF- α) [21–25].

CpG motifs may also be an explanation for the fact that intradermal or intramuscular injection of DNA induces a more reliable Th-1 response than the gene gun [26] because the amount of DNA applied in the injection mode is about 100 times higher compared to that of gene gun immunization. Therefore, Th-1 promotion is assumed to be the result of the high amount of plasmid DNA itself [27].

We have recently characterized the immune response against Bet v 1a after DNA immunization and the influence of CpG motifs [28]. In agreement with data dealing with the allergens Der p 5 [29], Hev b 5 [10] and the model molecule β -galactosidase [12], we found a predominant Th-1 type re-

sponse after intradermal injection of plasmid DNA encoding the allergen and a supporting effect of CpG motifs. However, recent data concerning DNA immunization and the effect of CpG motifs also point to an important influence depending on the nature of the protein itself [30].

Therefore, in the present paper we evaluated two isoforms of the allergen Bet v 1 with respect to their immunogenicity after DNA immunization in an animal system. According to their ability to bind IgE from allergic patients, Bet v 1 isoforms can be grouped into high-IgE-binding molecules and molecules with low/no IgE-binding activity. Bet v 1a belongs to the high-allergenicity group inducing wheal and flare reactions in the skin of birch-pollen-allergic individuals, whereas Bet v 1d revealed no such potency. On the other hand, the low-allergenic Bet v 1d isoform displayed significant higher T cell activation potency when compared to isoform a [31–33].

Materials and Methods

Construction of DNA Immunization Vectors

The following plasmids were constructed for DNA vaccination experiments: for pCMV-Bet a 489-bp fragment was amplified by PCR from the vector pMW175/Bet v 1a and pMW175/Bet v 1d [34]. The sequences of the 5' and 3' primers were 5'-GGTTGGAGAATTC-ATGGGTGTTTCAATTA-3' (*Eco*RI site underlined) and 5'-GGTTGGATCTAGATTAGTTGTAGGCATCGGAGTG-3' (*Xba*I site underlined) respectively, encompassing the entire Bet v 1a sequence. The PCR product was digested with *Eco*RI and *Xba*I and cloned into the eukaryotic expression vector pCI (Promega) and designated pCMV-Beta and pCMV-Betd, respectively. The expression cassette in pCI contains the CMV immediate-early promoter, a synthetic chimeric intron, a multiple cloning site, and the sv40 late poly(A) signal sequence.

A second type of expression vector was constructed by amplifying the Bet v 1a and d sequence from pCMV-Bet with the 5'*Eco*RI-containing primer mentioned above and a 3' primer 5'-CCCTCTAGATTAGACGTCAACGTCGACGTTAACGTTGTTGTAGGCATCGGAGTG-3'. This primer includes an *Xba*I restriction site (underlined), a stop codon (italic, bold) and 24 additional nucleotides. The attached nucleotides represent 4 different immunostimulatory CpG motifs (central CpG underlined, italics) which encode eight additional amino acids (NVNVDVDV) at the carboxyl terminal of Bet v 1a. The PCR product was cloned into *Eco*RI/*Xba*I-digested pCI vector. The resulting plasmids were denominated pCMV-Beta/app and pCMV-Betd/app.

The sequences of the constructs were verified using an Abi Prism™ Genetic Analyzer (Perkin Elmer, Norwalk, Conn., USA). Plasmid propagation was performed in *Escherichia coli* XI-Blue MRF' strain (Stratagene, La Jolla, Calif, USA). Purification of the plasmids was then performed using the Nucleobond AX-2000 Plasmid Prep (Macherey-Nagel, Bad Soden, Germany), according to the manufacturer's instructions. The quality and quantity of the DNA were analyzed by absorption at 260 and 280 nm and by agarose gel electrophoresis. The endotoxin content of the plasmids was <50 ng

LPS/mg DNA, as measured by Limulus amebocyte assay (Pyroquant, Walldorf, Germany).

Oligodeoxynucleotides

ODN were purchased from MWG-Biotech GmbH (Ebersberg, Germany). The sequence of the CpG-ODN was 5'-GCTAGACCGT-TAGCGT-3' (CpG motifs are underlined), the inverted CpG sequence was 5'-GCTAGAGCTTAGGCT-3'. The synthetic ODN were dissolved in LPS-free H₂O; the LPS content was <1 ng LPS/mg DNA.

Immunization and experimental groups

Female BALB/c mice, aged between 5 and 6 weeks, were obtained from the animal breeding facilities in Himberg, Austria, and maintained at the central animal care facility at the University of Salzburg according to the local guidelines for animal care. In all experiments, mice were bled before DNA injection to collect preimmune sera for antibody and cytokine assays. Sera were collected from mice by tail bleed. Sodium azide was added to a final volume of 0.2% and the sera were stored at 4°C. Prior to injection, the DNA vaccines were incubated at 95°C for 10 min and subsequently cooled at 4°C for 5 min. Mice were immunized intradermally into the shaved back (4 spots each) with 100 µg of plasmid DNA in 100 µl sterile PBS or with 100 µg plasmid DNA plus 50 µg of CpG-ODN in 100 µl PBS, respectively.

Groups of 6 mice were immunized with pCMV-Beta, pCMV-Beta/app, pCMV-Beta plus CpG-ODN (pCMV-Beta/coinj) and pCMV-beta/app plus CpG-ODN (pCMV-Beta/app/coinj) and with the respective vectors containing the Bet v 1d gene on days 1, 26, 55 and 90. Blood samples were taken on days 0 (preimmune serum), 25, 54, 75 and 120. Proliferation assays were done 4 weeks after the last booster injection.

Nonimmunized animals and animals immunized with the vector lacking the insert served as control groups. Additionally, mice were injected with Bet v 1a or Bet v 1d plasmid DNA together with an oligodeoxynucleotide with inverted CpG motifs (5'-GCTAGAGCTTAGGCT-3') and with CpG and GpC ODN alone.

Determination of Antigen-Specific Antibody Production by ELISA

For ELISA, black 96-well high-bind immunoplates (FluoroNunc, Nunc, Roskilde, Denmark) were coated by overnight incubation at 4°C with purified recombinant Bet v 1a or Bet v 1d protein (rBet, Biomay, Linz, Austria) at 1 µg/ml in PBS. Plates were washed with PBST using the AW1 automatic ELISA plate washing device (Anthos Labtec, Salzburg, Austria) and blocked with PBS containing 3% BSA for 2 h at 37°C. Sera were added 1:100 in PBS (1:20 for IgE detection) to the left column of each plate and serial 1:3 dilutions in PBS were then made in subsequent columns. Preimmune sera served as negative controls. The plates were incubated for 1 h at 37°C and then washed. Horseradish-peroxidase-conjugated detection antibody was added in PBS and incubated for 1 h at 37°C. For IgG1 and IgG2a detection, goat antimouse IgG1 or IgG2a (Pharmingen, San Diego, Calif., USA) was used at 1:2,000 dilution in PBS. Readings were referenced to commercial isotype standards, which were mouse IgG1 and IgG2a (Pharmingen).

For total IgG detection, goat-antimouse IgG(H+L) (BioRad, Germany) was used in the same way as for the subclass antibodies. The luminometric assay was developed with Luminol (BM chemiluminescence substrate, Boehringer-Mannheim, Germany) diluted 1:1 in H₂O. Chemiluminescence (photon counts/second) was determined using a LucyI Elisa-plate Luminometer (Anthos Labtec, Salzburg,

Austria). The obtained values were used to calculate the mean and standard error for each group of 6 mice.

Cytokine Detection in Sera and Supernatants of Stimulated Spleen Cells

IL-4 and IFN-γ were both measured by ELISA employing the multiple-antibody sandwich principle according to the protocol supplied by the manufacturer (Genzyme, Cambridge, Mass., USA). Briefly, 96-well microtiter plates (precoated with anti-mIFN-γ or anti-mIL-4 antibody) were incubated with standard and test samples. Supernatants of cultured spleen cells were used undiluted whereas serum samples were diluted 1:10 in PBS. To determine the absolute protein concentrations, standard calibration curves were obtained for each cytokine. After incubation with samples, a biotinylated anti-mIFN-γ or anti-mIL-4 antibody, which binds to the captured IFN-γ or IL-4 respectively, was added. Thereafter, the unbound antibody was removed by aspiration, plates were washed, and horseradish-peroxidase-conjugated streptavidin was added. After incubation, unbound material was removed by washing and the BM chemiluminescence substrate was added to the plates. The signals were luminometrically evaluated according to the procedure described above. The photon counts measured were proportional to the concentration of IFN-γ and IL-4 present in the standards or samples. A standard curve was obtained by plotting the concentrations of cytokine-standard versus the respective photon counts (the sensitivity was below 20 pg/ml). The cytokine concentrations were then determined using the standard curves.

Proliferation Assay of Isolated Spleen Cells

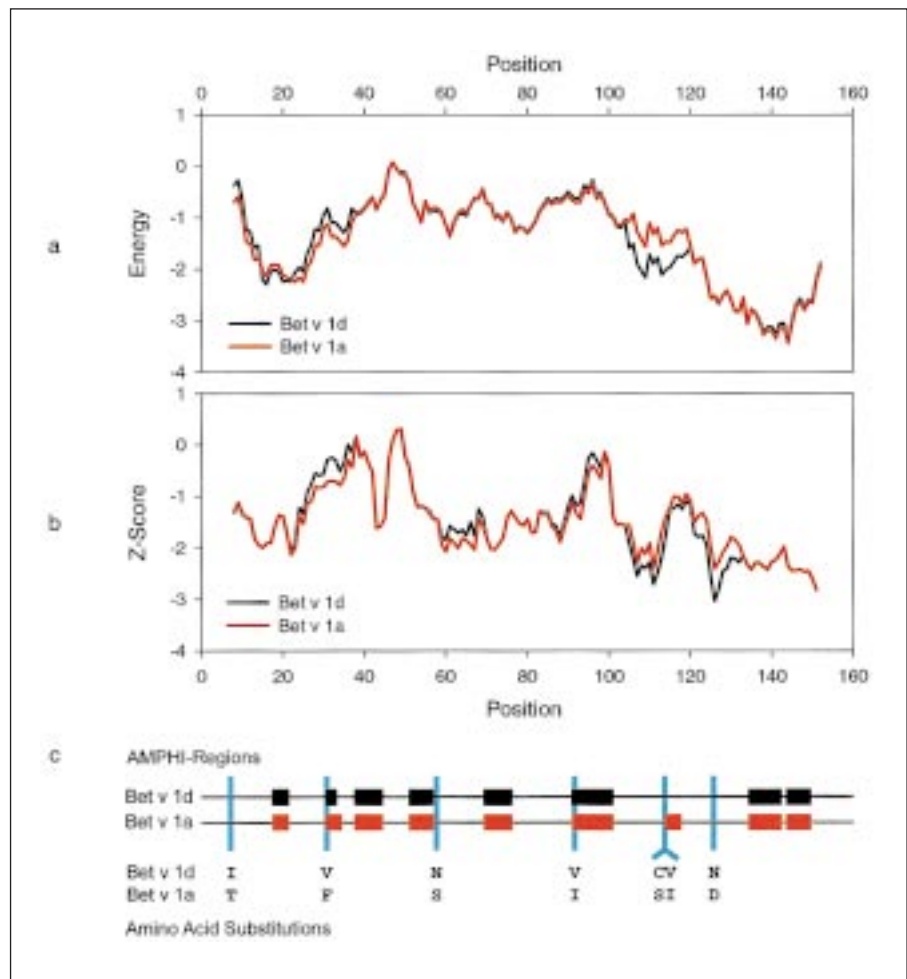
Spleens were minced in PBS, pH 7.2, and aggregated cells sedimented for 10 min. The supernatants containing a monodisperse cell suspension were washed 3 times in PBS and then resuspended in culture medium (DMEM) supplemented with streptomycin (100 µg/ml), penicillin (100 U/ml) and 1% heat-inactivated FCS. Cells (1×10⁶/well) were cultured with rBet v 1a and rBet v 1d, (20 µg/ml), respectively, in 96-well, flat-bottom, tissue culture plates for 52 h at 37°C, 95% RH, 7.5% CO₂. Cells were pulsed with 25 µCi/ml [³H]-thymidine (Amersham, UK) for an additional 20 h and then harvested with a cell harvester (Skatron, Lier, Norway). [³H]-thymidine incorporation was measured in a liquid scintillation counter (Beckman Coulter, Fullerton, Calif., USA).

Computer-Based Screening for T-Cell Epitopes

Immunodominant helper T lymphocyte antigenic sites were predicted from primary sequence data, using the AMPHI model of Margalit and Berzofsky [35]. The underlying assumption of the AMPHI method is that T cell antigenic sites are composed of amphipathic helices. The majority of known T cell epitopes contain amphipathic helices, however, it must be stated that this method will miss those that do not fit this premiss.

The calculation was done within the Protean module of the molecular biology Software-suite DNASTar. Amphipathic helices were predicted by first converting the primary amino acid sequence into hydrophobicity values, using the values of Fauchere and Pliska [35]. Second, the hydrophobicity sequence was divided into overlapping blocks of 7 residues (the parameter for proteins with low amphipathic content). Third, each hydrophobic block was searched for periodicity consistent with amphipathic helices. Finally, the program identified stable and unstable amphipathic helices. The results were displayed as an AMPHI region graph (fig. 1c).

Fig. 1. Computer based analysis of the two protein-isoforms Bet v 1a and Bet v 1d. Charts **a–c** utilize the same x-axis. **a** Comparison of energy plots (ProSA II plot) of Bet v 1d model with 1bv1. Differences in the shape of the energy curve indicate alterations of the folding. **b** Both folds (Bet v 1d model and 1bv1) were cut in overlapping fragments of a length of 15 amino acids. Z scores were calculated from that fragments and the values compared in order to detect differences in their properties. Lower Z scores point to a stabler fold. **c** In this AMPHI region graph, the position of potential T Lymphocyte antigenic sites are marked over the sequence. Amino acid substitutions among the protein isoforms are sketched out in blue.



Computational Analysis

A model for Bet v 1d was generated by aligning the sequence of Bet v 1d with 1bv1.pdb (Bet v 1a) [36, 37]. According to the alignment, the residues of Bet v 1d were mapped onto 1bv1 [38]. The energy plot of the Bet v 1d model was calculated (ProSA II plot) and compared with the energy plot of 1bv1 [39]. Differences in the shape of the energy curve indicate alterations of the folding which may be responsible for antigenic and/or immunogenic differences between the two isoforms (fig. 1a).

Fragmentation of folds and analysis/comparison of individual fragments were performed by cutting both folds (Bet v 1d model and 1bv1) in overlapping fragments. A length of 15 residues was chosen for the individual fragments because it represents the average size of peptides presented by MHC-II molecules. All possible fragments of this size were extracted and the Z scores calculated. The offset between the fragments was chosen to be one residue so that there are 14 overlapping residues between two consecutive fragments. The score was calculated for each fragment and the data used to generate a plot of Bet v 1d versus 1bv1 in order to detect differences between the two sequences (fig. 1b).

Statistical Analysis

Data are expressed as means $\pm$ SEM. Statistical significance was assessed using an unpaired Student's t test. All groups exhibited a normal distribution of the antibody, cytokine and stimulation values.

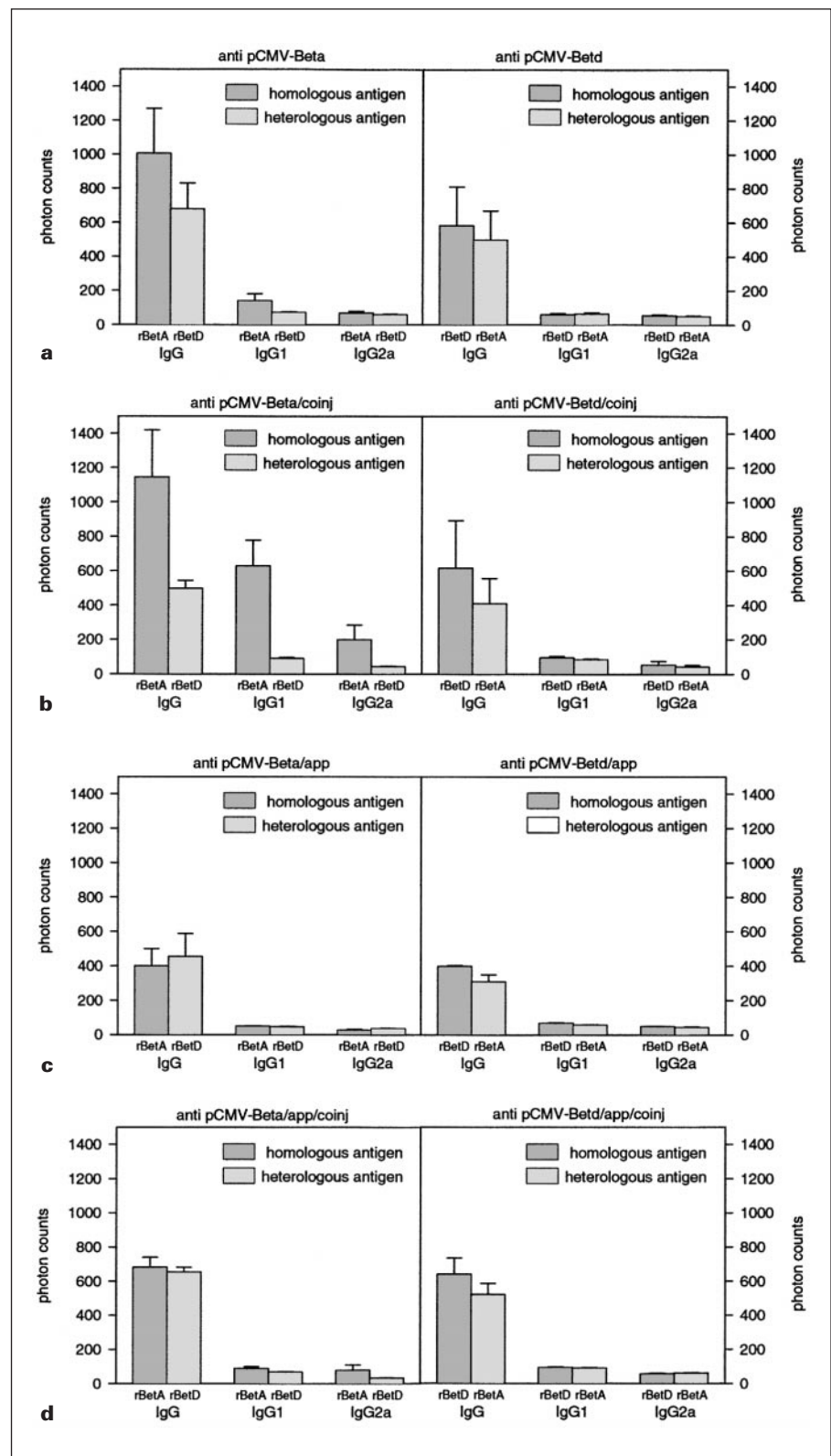
Results

Antibody Responses

Figure 2 shows the ELISA data of total IgG, IgG1 and IgG2a of animals immunized 3 times with allergen plasmid DNA and the cross-reactivity of antibodies with the heterologous allergen isoform.

Figure 2a: compared to pCMV-Beta, the pCMV-Betd plasmid induced a slightly lower antibody titer on homologous and heterologous antigen. Both antibodies against Bet v 1a and Bet v 1d cross-reacted with the heterologous antigens, pointing to a similar three-dimensional structure of both proteins. The levels of IgG2a were similar to those of

Fig. 2. ELISA of IgG, IgG1 and IgG2a immune responses after the third intradermal injection of plasmid DNA on plates coated with 100 ng/well recombinant Bet v 1a or Bet v 1d antigen. The dark columns represent ELISA data derived from homologous antigen (anti-Bet v 1a on Bet v 1a antigen; anti-Bet v 1d on Bet v 1d antigen), the light columns stand for ELISA data gained on heterologous antigen (anti Bet v 1a on Bet v 1d antigen; anti Bet v 1d on Bet v 1a antigen). Injections of pCMV-Bet(a,d) **(a)**, pCMV-Bet(a,d)/coinj with 50 μ g CpG-ODN **(b)**, pCMV-Bet(a,d)/app **(c)** and pCMV-Bet(a,d)/app/coinj with 50 μ g CpG-ODN **(d)** were given at days 1, 26 and 55. Blood was taken at day 75 and the sera for this experiment were diluted 1:100. Data are expressed as mean $\pm$ SEM.



IgG1. Because typical Th-2 responses after allergen-protein immunization are characterized by a ratio >10 for IgG1, these data in principle indicated a predominant Th-1 type response against both isoforms. A more stringent indication for a Th-1 response came from the fact, that no IgE could be detected at any time of the immunization schedule (data not shown).

Figure 2b: to study the influence of CpG-motifs on antibody responses, coinjection of CpG-ODN together with plasmids encoding Bet v 1a or Bet v 1d (designated pCMV-Beta/coinj and pCMV-Betd/coinj) were performed. Coinjection of CpG-ODN resulted in an approximately threefold titer of IgG1 and IgG2a in the group immunized with pCMV-Beta/coinj on homologous Bet v 1a antigen. Furthermore, this group revealed a statistically significant difference ($p < 0.05$) between the homologous (on Bet v 1a) and heterologous reaction (on Bet v 1d), which may reflect subtle antigenic differences between the two isoforms or an antigen-depending CpG effect [30]. The humoral response against Bet v 1d was not influenced by coinjection of CpG motifs.

Figure 2c, d: two additional approaches testing the role of immunostimulatory CpG motifs were performed: (1) inclusion of CpG motifs into the gene of both Bet v 1a and Bet v 1d by appending 4 CpG motifs to the coding sequence (termed pCMV-Beta/app and pCMV-Betd/app, respectively); (2) coinjection of CpG-ODN together with either pCMV Beta/app or pCMV Betd/app plasmid DNA (designated pCMV-Beta/app/coinj and pCMV-Betd/app/coinj). CpG motifs, both appended and appended plus coinjected, reduced the antibody titers against Bet v 1a. The humoral response against Bet v 1d was not influenced by any application of CpG motifs.

Immunization with plasmid DNA together with inverted CpG-ODN (5'GCTAGAGCTTAGGCT-3') did not result in any significant changes of the immune response compared to immunization with pCMV-Beta or pCMV-Betd. Injection of CpG- and GpC-ODN alone exhibited no antigen-specific responses (data not shown).

Structural Analysis of Bet v 1a and Bet v 1d

Since the homology of both proteins is more than 95%, a similar folding was expected. In general, this assumption was confirmed by computing an energy plot of both structures using ProSA II [39] (fig. 1a). However, the plot also showed a remarkable difference in the region of residues 100–120. In this region, two residues differ between Bet v 1d and Bet v 1a (namely Ser $\rightarrow$ Cys 112 and Ile $\rightarrow$ Val 113). It seems obvious that the discrimination in the energy curve was caused by these exchanges. Z-scores used as a quality

measure for the complete fold [38] were calculated for both folds, model and native Bet v 1a (1bv1). The values were highly similar and in a range as expected for correctly folded proteins (data not shown).

Appending of CpG motifs, encoding for 8 additional amino acids to the carboxyl terminal elicited no significant alteration of the structure concerning the energy plot and Z-scores compared to their protein ancestors (data not shown).

In principle, the structural analysis pointing to a very similar folding of the investigated proteins is in agreement with the experimental data obtained in antibody cross-reaction ELISAs.

Serum Cytokine Profiles

In order to characterize the response type against both allergens concerning a predominant Th-1 or Th-2 reaction more precisely, the key cytokines IFN- γ and IL-4 were measured in the serum of immunized animals during the course of the whole immunization schedule.

Surprisingly, Bet v 1a and Bet v 1d induced a strikingly different production of both cytokines (fig. 3). Immunization with pCMV-Beta and its modifications clearly stimulated a strong and typical Th-1 response including elevated IFN- γ and reduced IL-4 production. Despite a negligible humoral response after the first injection (data not shown), the cytokine profiles pointed to an effective cellular primary response, even resulting in a systemic alteration of the key cytokines, with the most pronounced response following upon immunization with pCMV-Beta/coinj.

The highest ratio of IFN- γ to IL-4 was measured for the pCMV-Beta/coinj group in the primary response, and for all other groups after the second injection.

After a third injection of plasmid DNA, the cytokine ELISA yielded values similar to the pre-sera for all groups. This normalization of serum cytokines had no effect on the antibody titers, which still increased after the third injection.

Both Bet v 1a groups coinjected with CpG motifs elicited a slightly stronger IL-4 suppression; however, these differences proved not to be statistically significant.

The cytokine profiles of all groups injected with plasmid DNA encoding for Bet v 1d revealed no statistically significant alteration of IFN- γ and IL-4 after the first injection (a tendency of IL-4 decrease can be seen after immunization with pCMV-Betd and pCMV-Betd/coinj). In contrast to the Bet v 1a groups, a drastic increase of IL-4 was induced after the second injection of plasmid DNA in all groups. A third injection led again to normalization of the IL-4 values.

Also in contrast to all Bet v 1a groups, no increase of IFN- γ was detectable in any group at any time during the

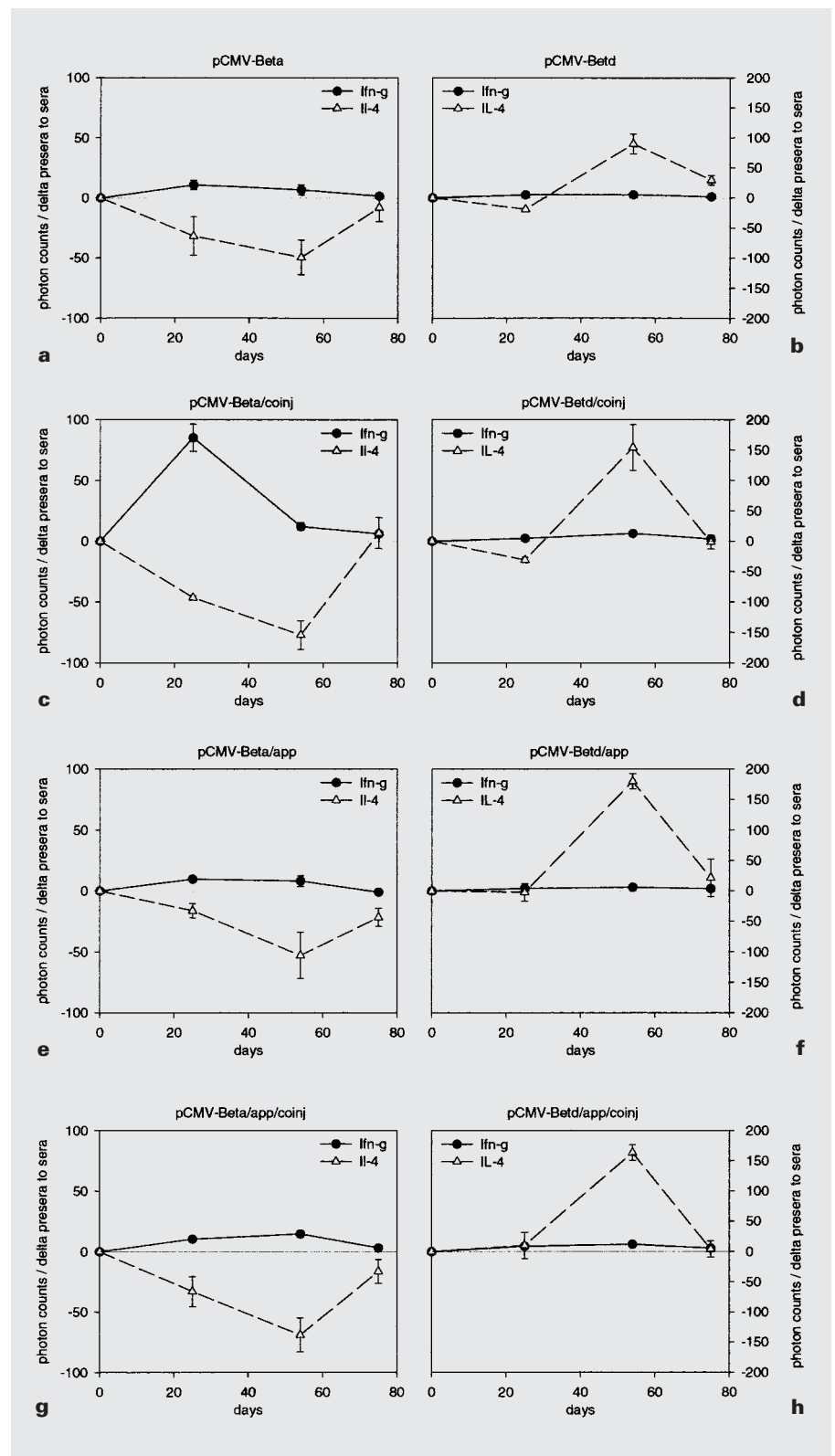


Fig. 3. Cytokine ELISA of IFN- γ and IL-4 in serum of animals ($n = 6$) immunized with pCMV-Bet(a,d) (**a**, **b**), pCMV-Bet(a,d)/coinj coinjected with 50 μ g CpG-ODN (**c**, **d**), pCMV-Bet(a,d)/app (**e**, **f**) and pCMV-Bet(a,d)/app/coinj coinjected with 50 μ g CpG-ODN (**g**, **h**). Injections were given at days 1, 26, 55 and blood samples were taken on days 0 (preimmune serum), 25, 54 and 75. Preserum values were subtracted from experimental measures to generate delta values (absolute increase/decrease of cytokine levels). Preserum values for IFN- γ and IL-4 were between 3 and 8 pg/ml. Data are expressed as mean $\pm$ SEM.

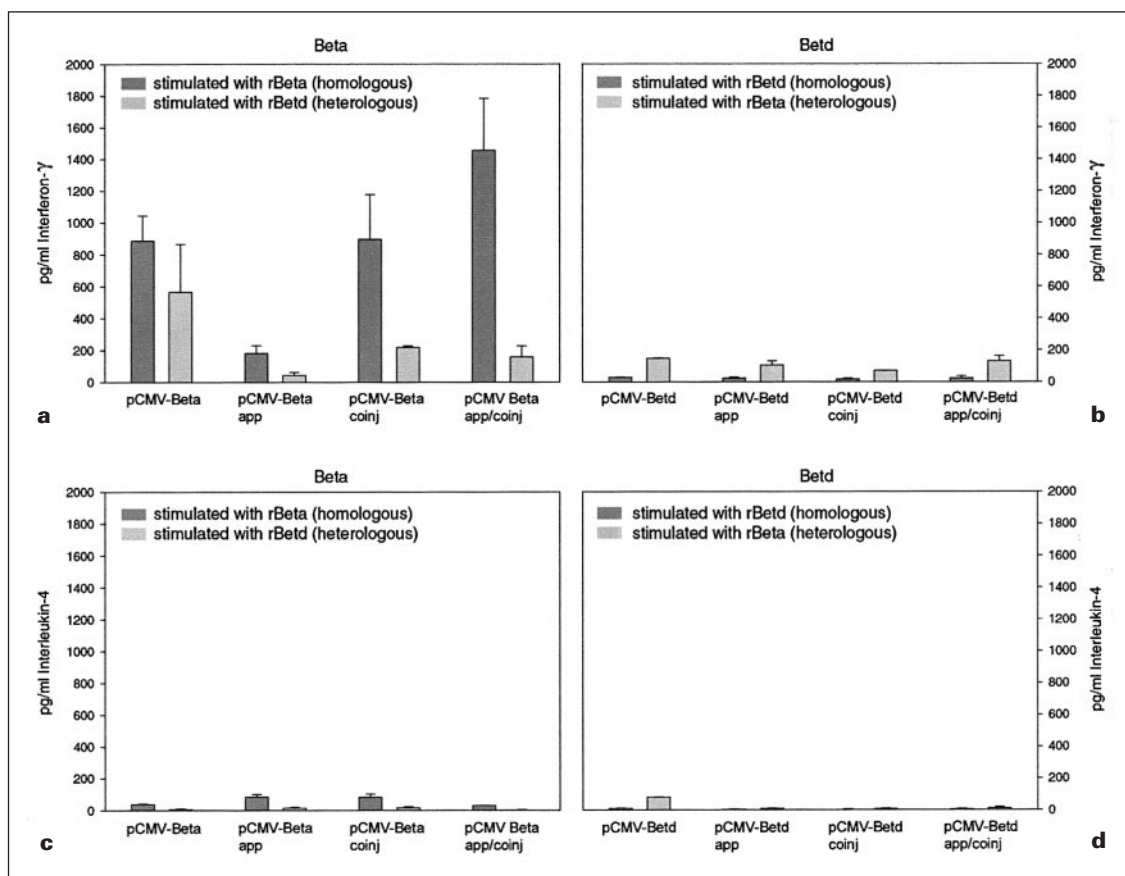


Fig. 4. Cytokine ELISA of IFN- γ (a, b) and IL-4 (c, d) in undiluted supernatants of proliferation cultures 30 days after four monthly injections. Spleen cells were stimulated with either homologous or heterologous protein-antigen. Data are expressed as mean $\pm$ SEM. IL-4 suppression was highly significant in all groups ($p < 0.001$).

course of the experiment. Furthermore, additional CpG motifs did not alter the cytokine levels in any group.

These results seem to indicate the induction of a more pronounced Th-2 type response by Bet v 1d plasmid DNA. However, on the other hand this seems to be in contradiction to the fact that no increase of IgG1 could be measured in any group and, moreover, no IgE production was detectable at any time.

Cytokine Profiles in Supernatants of Allergen-Stimulated Spleen Cells

Antigen-specific proliferation was performed and the culture supernatants analyzed for IFN- γ and IL-4 (fig. 4). As a time point, 4 weeks after the third injection of DNA was chosen, which should enable us to answer some important questions: Was the response type stable over a prolonged period and after repeated boosting, and was a mem-

ory established? These questions arose because after three injections both IFN- γ and IL-4 levels normalized, and one could argue that the observed response types had been only temporary.

Immunization with all Bet v 1a plasmids revealed a significant IFN- γ to IL-4 ratio in culture supernatants of spleen cells stimulated with rBet v 1a (an approximately 2.5- to 50-fold excess of IFN- γ in the different groups), indicating a very strong Th-1 response (with the highest ratio for the group immunized with pCMV-Beta/app/coinj). Heterologous stimulation with an equivalent amount of rBet v 1d significantly decreased cytokine expression, however, still in a Th-1 typical mode (an approximately 3- to 150-fold excess of IFN- γ in the different groups).

The evaluation of both key cytokines from animals immunized with plasmid DNA encoding for Bet v 1d and stimulated with rBet v 1d (homologous stimulation) elicited

an approximately 5-fold reduction in cytokine expression in all groups. Nevertheless, the ratio of IFN- γ to IL-4 clearly pointed to a Th-1 response. IL-4 was slightly suppressed in all animals treated with CpG motifs.

In contrast to the Bet v 1a groups, heterologous stimulation of spleen cells from animals immunized with Bet v 1d with rBet v 1a resulted in higher expression of both IFN- γ and IL-4 than in the homologous approach. As in the homologous situation, the ratio of IFN- γ and IL-4 indicates a Th-1-type response with the exception of the group pCMV-Betd. Only in this group does IL-4 production (150 pg/ml versus 79 pg/ml) seem not be strikingly suppressed, which may be a result of lacking CpG motifs in the immunization protocol.

In order to test the antigen specificity of the response, all experimental groups were also stimulated with the nonrelated antigens myoglobin and ovalbumin. Both antigens did not induce either IFN- γ or IL-4, thus indicating the specificity of the response.

Proliferative Responses in Allergen-Stimulated Spleen Cells

In principle, the results obtained by determination of the cytokines from proliferation cultures were confirmed by the antigen-specific stimulation of spleen cells as measured by ^3H -thymidin incorporation (fig. 5). Animals immunized with pCMV-Beta/coinj and pCMV-Beta/app/coinj elicited the highest stimulation values ($p < 0.005$) pointing to a supporting role of CpG motifs for cellular reactions. No significant stimulation could be detected in the groups immunized with pCMV-Betd and its modifications. This was also true for the heterologous approach (animals immunized using Bet v 1d plasmid DNA stimulated with rBet v 1a). In this case, it must be considered that the stimulation of non-immunized controls with rBeta was nearly tenfold compared to rBetd. Therefore, these values must be related to the rBeta control.

In contrast, the reverse heterologous approach demonstrated that spleen cells of animals immunized with Bet v 1a plasmid DNA proliferated after stimulation with rBetd (again with a significant increase in the pCMV-Beta/coinj and pCMV-Beta/app/coinj groups; $p < 0.005$).

Stimulation with the nonrelated proteins myoglobin and ovalbumin induced no proliferation in any group.

T Cell Epitopes and Z Scores of Fragmented Folds

Utilizing the AMPHI-algorithm Bet v 1a exhibits one additional helper T Lymphocyte antigenic site compared to Bet v 1d at position 114–117. Interestingly, the fragments surrounding this position also show differences in their Z

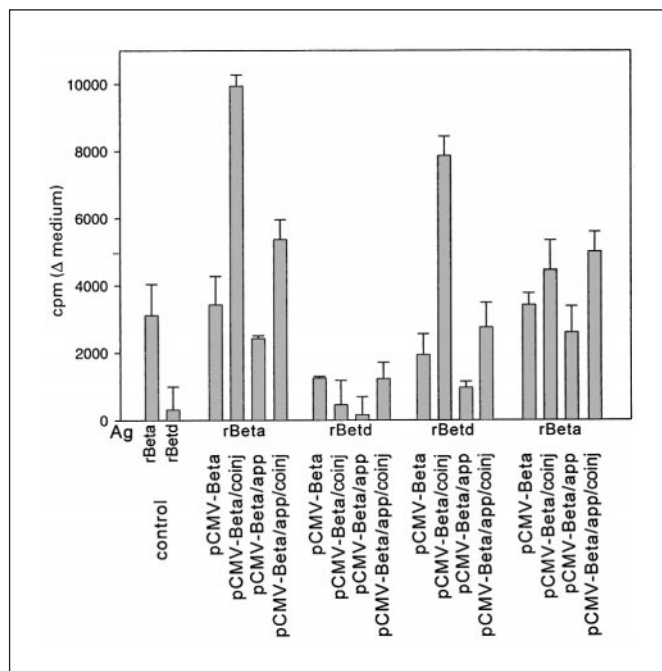


Fig. 5. Allergen-specific proliferation of isolated spleen cells. Spleen cells of controls and immunized animals were cultured for 52 h with recombinant Bet v 1a or recombinant Bet v 1d and pulsed with 25 $\mu\text{Ci/ml}$ ^3H -thymidine for 20 h. Values are expressed in Δcpm (medium), mean $\pm$ SEM.

scores and in the energy plot. The amphipathic helix at position 31–34 in Bet v 1a exceeds the corresponding helix in Bet v 1d by one residue (fig. 1c). This second T cell epitope alteration is also reflected in Z score differences around this position (fig. 1b).

Discussion

Numerous controlled clinical studies have demonstrated the efficacy of specific immunotherapy (SIT) of type I allergy as a treatment for patients suffering from pollinosis [40, 41]. However, at present, there is only a general agreement for SIT in the treatment of venom hypersensitivity and allergic rhinitis. Its usefulness and safety for treating food allergy and asthma are still discussed [42, 43] and the occurrence of typical allergic symptoms after injection of allergens into sensitized organisms is frequent. Due to these unwanted side effects, therapeutic doses can sometimes not be reached, and SIT has to be considered a potentially dangerous therapy, which, moreover, is not successful in some cases. Recently, evidence has accumulated that SIT influ-

enced allergen-specific T helper cell responses [44–47]. From these studies, it can be concluded that a modulation of the allergen-specific immune reactivity from a Th-2 oriented to a Th-1-biased response is the operating mechanism leading to clinical success. Based on these findings, several strategies of inducing Th-1-like immune responses to allergens have been established [12, 48, 49].

Immunization with plasmid DNA, a novel approach in vaccine research and development, offers several advantages for a new type of allergen-specific immunotherapy. Because of the minimal concentration of antigen (only nanograms of proteins are translated at the injection site), as compared to the doses used for conventional immunotherapy [9], no side effects like inflammatory responses or anaphylactic reactions are caused by plasmid DNA. Furthermore, DNA can be highly purified, thus containing no contamination such as recombinant proteins may frequently exhibit.

DNA immunization with clinically relevant Bet v 1 proteins not only opens new prospects for an alternative immunotherapy, but also can address the question as to what extent the nature of the molecule specifically influences the immune reaction. The isoforms Bet v 1a and Bet v 1d are excellent candidates for such a question. In human studies, the former exhibited a strong allergenic nature, with high levels of specific IgE antibodies in about 80% of the individuals allergic to birch pollen, whereas the latter is assumed to be only weakly allergenic, but with a stronger capacity to activate T cells. Therefore, Bet v 1d was proposed for a new form of specific immunotherapy using hypoallergenic recombinant allergen isoforms [31–33].

Computer analysis performing energy blots (ProSA II Plots) and Z scores of Bet v 1a and Bet v 1d and their descendants (Beta/app and Betd/app) revealed a very similar folding, which only differed in the region 100–120.

In principle, these data were also confirmed by the fact that antibodies produced against isoform Bet v 1a cross-reacted in a comparable manner with the isoform Bet v 1d and vice versa. Immunization with plasmid DNA of both isoforms also induced a similar level and type of humoral response (indirectly pointing to a similar expression level of both isoforms) with respect to the isotypes and subclasses indicating a predominant Th-1 response type for both molecules.

Minor differences concerning B cell epitopes cannot be excluded because immunization with plasmid DNA encoding the native protein resulted in a decreased cross-reaction. In contrast, this effect could not be detected in the groups with appended CpG motifs. As described in Materials and Methods, the mode of appending immunostimulatory se-

quences leads to additional translation of amino acids at the carboxyl terminal of the allergen. This slight modification seems to have no drastic influence on the three-dimensional structure in molecular modeling. However, it may be sufficient to introduce subtle antigenic alterations.

No major variations were observed in the distribution of IgG1 and IgG2a subclasses during the whole immunization schedule. Both subclasses were detectable with a maximum ratio of 3:1 for IgG1. Considering a ratio of IgG1 to IgG2a >10 as an indicator for a typical Th-2-type response after genetic immunization [26], the subclass distribution also indicated a Th-1-type response. However, it should be pointed out that the expression of subclasses (without measuring the key cytokines) cannot be taken as a reliable indication of either a Th-1- or a Th-2-type reaction [50].

Additional strong evidence for a Th-1 response against both isoforms stems from the fact that no IgE antibodies could be detected after genetic immunization in any of the animals at any time during the experiments. This is in agreement with data reported after DNA immunization of mice with a plasmid construct containing the latex allergen Hev b 5, and with plasmid DNA encoding Der p 5, an important house dust mite allergen [10, 11].

The determination of serum cytokines during the whole course of the immunization schedule, however, elicited striking differences between the two isoforms. The reaction against Bet v 1a was clearly a Th-1-type response with elevated IFN- γ (especially in the pCMV-Beta+CpG group) and decreased IL-4 values after two injections. In contrast, during the response against Bet v 1d no increase in IFN- γ was observed, but one marked peak of IL-4 after the second injection of plasmid DNA was measured. Both values normalized after a third injection of plasmid DNA, but with no correlation with the antibody titers, which increased after each booster injection. This lack of correlation of serum cytokines and antibody titers has been recently demonstrated for the course of the immune reaction against plasmid DNA encoding Bet v 1a [28].

An important question arose from these data: does the IL-4 peak during the response against Bet v 1d reflect a true Th-2-type reaction? For this purpose the key cytokines were measured in supernatants of proliferation cultures 4 weeks after the last injection. The results revealed that both isoforms induced a significantly higher IFN- γ than IL-4 production, thus also pointing to a Th-1 reaction against Bet v 1d. The difference between both isoforms in these experiments was restricted to the fact that rBet v 1a induced a strikingly higher amount of both IFN- γ and IL-4. In principle, the cytokine data correlated well with the results of the proliferation experiments. Animals immunized with Bet v

1a plasmid DNA elicited the highest reactivity (for the key cytokines and stimulation values) in all cases, both in the homologous (immunized with Bet v 1a and stimulated with rBet v 1a) and in the heterologous (immunized with Bet v 1a and stimulated with rBet v 1d) approach.

Comparing these data from animal experiments with data from human T cell lines, T cell clones and IgE-binding experiments [32, 33, 51], the concurrence is restricted to the fact that both isoforms generally differ in their induction of cellular immune responses. Furthermore, the differences between the serum cytokine profiles indicated different systemic responses.

The molecule Bet v 1d itself proved to have no toxic effects in proliferation cultures (as it could be concluded from the homologous proliferation experiment) because it was able to stimulate spleen cells and induce cytokine expression from animals immunized with Bet v 1a plasmid DNA.

Apart from a slight suppression of the antibody production, which has also been demonstrated by recent publications [30, 52], CpG motifs do not seem to primarily affect the humoral response but have more influence at the cellular level. This is in agreement with the assumption that CpG motifs exert their function via activation of cells and cytokines of the innate immune system [21–23]. The results from antigen-specific stimulation experiments clearly demonstrate that additional and combined application of CpG motifs strongly effects the cellular responses. In the supernatant of spleen cells stimulated with rBet v 1a a marked increase of the production of IFN- γ and the highest degree of IL-4 suppression was detected in the group pCMV-Beta/app+CpG. This IL-4 suppressive effect could also be clearly seen in the heterologous approach using spleen cells from animals with Bet v 1d immunized treated with rBet v 1a. The animals immunized with pCMV-Bet v 1d plasmid DNA, which received no additional CpG motifs, showed the highest IL-4 titers.

At present, we have no explanation for the different cellular reaction patterns of Bet v 1a and Bet v 1d. One possibility could be that the minimal sequence alterations of the isoform d lead to different processing products with a reduced T-cell activation potency. This seems to be confirmed by the computer-based screening for T cell epitopes, calculation of energy plots and Z scores, which concurrently exhibited differences between the isoforms in the sequence region 100–120. Proliferation assays with overlapping peptides should be able to clarify this question.

Summing up the results, we can state that plasmid DNA of both isoforms of Bet v 1 induced similar humoral responses. Both the subclass composition, the lack of IgE production and the cytokine profiles from supernatans of stim-

ulated spleen cells pointed to a clear Th-1-type reaction. However, the isoforms strikingly differed in their immunogenicity with respect to allergen-specific proliferation and in the serum cytokine profile during the immune response. Further experiments with the isoform Bet v 1d must be performed to answer the question whether the suppression of the proliferative response, which also includes the response against the heterologous isoform, can prevent an allergic reaction (possibly by decreasing the allergen-specific T-helper cell expansion).

Nevertheless, the present data seem to favor DNA-based immunotherapy with the isoform Bet v 1a coinjected with CpG motifs because this combination strongly supports the development of a Th-1-type response. It remains to be established whether an immunization with a Bet v 1 gene can prevent already established atopic immune responses, and downregulate an ongoing Th-2 response. Additionally, the results also confirm the assumption that the antigen itself [30] can have a marked influence on the immune response after genetic immunization.

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