

Immune responses after immunization with plasmid DNA encoding Bet v 1, the major allergen of birch pollen

Arnulf Hartl, MSc,^a Jan Kiesslich, MSc,^a Richard Weiss, MSc,^a Andrea Bernhaupt, MSc,^a Sven Mostböck, MSc,^a Sandra Scheiblhofer, MSc,^a Christof Ebner, MD,^b Fátima Ferreira, PhD,^c and Josef Thalhamer, PhD^a Salzburg and Vienna, Austria

Background: Immunization with plasmid DNA encoding various antigens is a promising method in vaccine research. Recent studies also indicate that DNA-based immunization might represent a potential approach in allergen-specific immunotherapy. **Objective:** In this study we have characterized the immune responses induced by recombinant Bet v 1a and plasmid DNA encoding for Bet v 1a, the major allergen of birch pollen in a mouse system.

Methods: Balb/c mice were injected intraperitoneally with recombinant Bet v 1a and intradermally with plasmid DNA encoding for the gene of Bet v 1a (pCMV-Bet). In addition, the effect of immunostimulatory DNA sequences was investigated by appending CpG motifs to the gene of Bet v 1a, coinjecting CpG-oligodeoxynucleotides together with the pCMV-Bet construct, or both. IgE and IgG antibody responses, as well as IgG subclasses, were measured by ELISA in sera after each immunization. IFN- γ and IL-4 levels were also measured by ELISA in sera and supernatants of allergen-stimulated spleen cells.

Results: The primary humoral response to a single treatment with pCMV-Bet was very weak, but the reaction could be boosted to higher levels by 2 additional injections. On the other hand, proliferation assays of spleen cells and measurements of cytokine levels already indicated a cellular response after the first injection of plasmid DNA. After 2 immunizations with pCMV-Bet, the ratio of IgG1 to IgG2a pointed to a T_{H1} subclass profile. IgE was not detectable in any group at any time during the immune reaction. Accordingly, IL-4 levels were markedly reduced in the serum, as well as in the supernatants, of stimulated spleen cells. Animals immunized with pCMV-Bet containing appended CpG motifs at the 3' end of the Bet v 1a gene and/or with the CpG-ODN GCTAGACGT-TAGCGT plus pCMV-Bet displayed reduced humoral responses against Bet v 1a when compared with animals injected with pCMV-Bet alone. The levels of IFN- γ measured after allergen stimulation of isolated spleen cells were significantly higher in animals immunized with pCMV-Bet plus CpG motifs than

with pCMV-Bet alone. Immunization with recombinant Bet v 1a protein elicited a strong T_{H2}-type response, including IgE production, a high titer of IgG1, and IL-4 production in both serum and supernatants of proliferation cultures.

Conclusion: In contrast to immunization with protein, DNA immunization induces a strong T_{H1}-type response against a relevant inhalant allergen. Our data support the concept of developing a novel type of allergen immunotherapy based on plasmid DNA immunization. (*J Allergy Clin Immunol* 1999;103:107-13.)

Key words: Bet v 1, DNA immunization, birch pollen allergen, immunotherapy, Balb/c

The observation that the injection of plasmid DNA encoding an antigen induces synthesis of the respective protein and a specific immune response led to intensive research for the potential use of plasmid DNA in vaccine development. Since then, the induction of immune responses against a wide variety of antigens from infectious agents like viruses, parasites, and bacteria has been demonstrated by immunization with plasmid DNA.¹ Furthermore, both the delivery of DNA to a tissue by means of a gene gun and injection of a saline-DNA solution intramuscularly or intradermally proved to induce antigen-specific humoral and cellular responses.²⁻⁵ Presently, there is a broad consensus in respect to the mechanisms of DNA-based immunization and the basic events after an injection of plasmid DNA.¹ In contrast to immunization with proteins, which are taken up by antigen-presenting cells (APCs) and consequently presented by means of MHC-II, plasmid DNA must first transfect cells to be translated into proteins. The predominant pathway therefore goes through MHC-I molecules, thus leading to the activation of a cellular immune response. However, the separation of both aspects of the immune system in DNA-based immunization is not that strict because translated proteins are also available for APCs to induce humoral responses.⁶⁻⁸

The major difference between the immune response elicited by conventional immunization with protein antigens and DNA is the induction of a predominant T_{H1}-type response. One possible explanation for this phenomenon may be the presence of unmethylated CpG dinucleotides flanked by 2 5' purines and 2 3' pyrimidines in the sequences, which were shown to stimulate the innate immune system to produce a number of T_{H1}-inducing cytokines (including IL-12, IL-18, IFN- α , β , and TNF- α).⁹⁻¹¹ These so-called immunostimulatory CpG motifs are 20 times as common in bacterial DNA than in mammalian DNA, and may serve as a "danger signal" for the

From ^athe Institute of Chemistry and Biochemistry, University of Salzburg;

^bthe Institute of General and Experimental Pathology, University of Vienna; and ^cthe Institute of Genetics and General Biology, University of Salzburg.

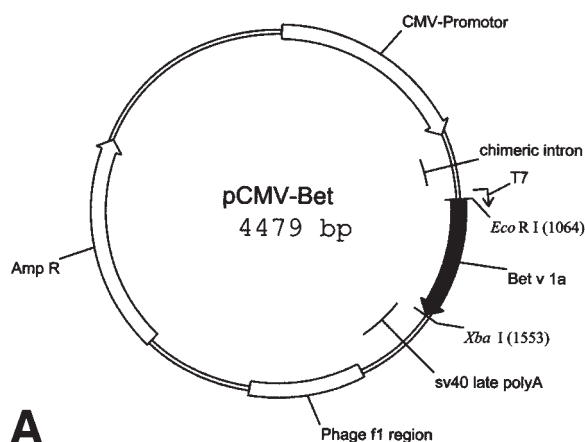
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Reprint requests: Josef Thalhamer, PhD, Institute of Chemistry and Biochemistry, University of Salzburg, Hellbrunnerstr. 34, A-5020 Salzburg, Austria.

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**A**

TyrAsnAsnValAsnValAspValAspVal***

CpG 2 CpG 4

CpG 1 CpG 3

TACAACAACGTTAAACGTCGACGTTGACGTTCTAA

ATGTTGTTGCAATTGCAGCTGCAACTGCAGATT

B

FIG 1. Expression vector pCMV-Bet and pCMV-Bet/app. The expression cassette contains the CMV immediate-early promoter, a synthetic chimeric intron, a multiple cloning site, and the sv40 late poly(A) signal sequence (**A**). pCMV-Bet/app was constructed by attaching 4 different immunostimulatory CpG motifs at the carboxy-terminus of Bet v 1a, which encode 8 additional amino acids (**B**).

Abbreviations used

APC: Antigen-presenting cell
ODN: Oligodeoxynucleotide
SIT: Specific immunotherapy

mammalian immune system. Most plasmids frequently used for DNA-based immunization contain the Amp resistance gene and the CMV promoter sequence. CpG motifs may also be an explanation for the fact that intradermal or intramuscular injection of DNA induces a more reliable T_{H1} response than the gene gun¹² because the amount of DNA applied in the injection mode is about 100 times higher compared with that of the gene gun immunization. Therefore T_{H1} promotion is assumed to be the result of the high amount of the plasmid DNA itself.¹³

Type I allergy is a widespread and increasing phenomenon affecting approximately 20% of the population in industrialized countries. One of the characteristics of allergic diseases is the permanent production of IgE molecules against common environmental antigens,¹⁴ which is mainly dependent on IL-4 and inhibited by INF- γ . On the basis of animal studies, not only CD4⁺ cells, but also CD8⁺ T cells, seem to play a regulatory role in allergic responses.^{15,16}

Recently, it has been suggested that intradermal vaccination with plasmid DNA might find a potential therapeutic application as a new form of allergen-specific immunotherapy.^{17,18} Therefore the aim of this study is to characterize the immune response elicited by protein and DNA-based immunization against a clinically relevant allergen, namely Bet v 1a, and the influence of CpG motifs on humoral and cellular reactions. This 17.4-kd protein represents the major allergen of birch pollen and induces specific IgE responses in more than 95% of patients allergic to birch pollen (60% of patients allergic to tree pollen react exclusively to this protein).^{19,20} Moreover, Bet v 1a shows extensive cross-reactivity with homologous proteins from the pollen of related trees and foodstuffs.²¹

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METHODS

Construction of DNA immunization vectors

Two plasmids were constructed for DNA vaccination experiments (Fig 1, A and B). For pCMV-Bet, a 489-bp fragment was amplified by PCR from the vector pMW175/Bet v 1a.²² The sequences of the 5' and 3' primers were 5'-GGTTGGA-GAATTCATGGGTGTTT-CAATTA-3' (*EcoRI* site underlined) and 5'-GGTTGGATCTAGATTAGTTGTAGGCATCG-GAGTG-3' (*XbaI* site underlined), respectively, encompassing the entire Bet v 1a sequence. The PCR product was digested with *EcoRI* and *XbaI* and cloned into the eukaryotic expression vector pCI (Promega) and designated pCMV-Bet (Fig 1, A). 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 expression vector was constructed by amplifying the Bet v 1a sequence from pCMV-Bet with the 5' *EcoRI*-containing primer mentioned above and a 3' primer 5'-CCCTCTAGATTAGACGTCACGTCGACGTTAACGTTGTTG-TAGGCAT-CGGAGTG-3'. This primer includes an *XbaI* restriction site (underlined), a stop codon (italic, bold), and 24 additional nucleotides. The attached nucleotides represent 4 different immunostimulatory CpG motifs (central CpG underlined, italic), which encode 8 additional amino acids (NVNVDVDV) at the carboxy-terminus of Bet v 1a. The PCR product was cloned into *EcoRI/XbaI*-digested pCI vector. The resulting plasmid was designated pCMV-Bet/app (Fig 1, B).

Both plasmids were sequenced with a Thermo-Sequenase Cycle Sequencing protocol (Amersham, Buckinghamshire, UK). Plasmid propagation was performed in *Escherichia coli* XL-1Blue MRF' strain (Stratagene, La Jolla, Calif). Purification of the plasmids was then performed by 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. Endotoxin content of the plasmids was less than 50 ng LPS/mg DNA, as measured by Limulus amoebocyte assay (Pyroquant, Walldorf, Germany).

Oligodeoxynucleotide

The oligodeoxynucleotides (ODNs) were purchased from MWG-Biotech GmbH (Ebersberg, Germany). The sequence of the CpG-ODN was 5'-GCTAGACGTTAGCGT-3' (CpG motifs are underlined). The synthetic ODN was dissolved in LPS-free H₂O; the LPS content was less than 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

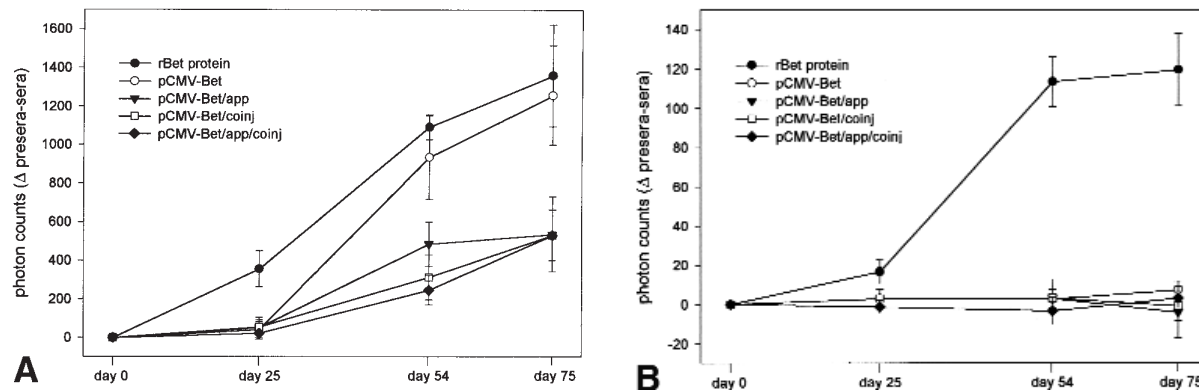


FIG 2. ELISA of IgG (**A**) and IgE (**B**) immune responses after intradermal injection of pCMV-Bet, pCMV-Bet/app, pCMV-Bet/coinj with 50 μ g CpG-ODN, and pCMV-Bet/app/coinj with 50 μ g CpG-ODN and intraperitoneal injection of 1 μ g rBet v 1a. Injections were given at days 1, 26, and 55, and blood samples were taken on days 0 (preimmune serum), 25, 54, and 75 ($n = 6$ for each group). Recombinant Bet v 1a served as antigen; serum dilution used for this graph was 1:100 (IgG) and 1:20 (IgE). Data are expressed as mean $\pm$ SEM. Only marginal antibody levels were induced by the first injection of plasmid DNA; boosting led to antibody production similar to that after immunization with rBet v 1a. Both means of immunostimulatory sequence application decreased the IgG response significantly ($P < .05$) in all 3 groups after the second and third injection. IgE antibodies were detectable only after immunization with the recombinant allergen.

experiments mice were bled before protein or 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. Before injection, the DNA vaccines were incubated at 95°C for 10 minutes and subsequently cooled at 4°C for 5 minutes. Mice were immunized intradermally into the shaved back (4 spots each) with 100 μ g of plasmid DNA in 100 μ L sterile PBS or with 100 μ g plasmid DNA plus 50 μ g of CpG-ODN in 100 μ L PBS, respectively. Intraperitoneal immunization with recombinant Bet v 1a protein was performed with 1 μ g protein in 50 μ L H₂O plus 100 μ L 2% Al(OH)₃ solution (Serva, Vienna, Austria).

Groups of 6 mice were immunized with recombinant Bet v 1a protein, pCMV-Bet, pCMV-Bet/app, pCMV-Bet plus CpG-ODN (pCMV-Bet/coinj), and pCMV-Bet/app plus CpG-ODN (pCMV-Bet/app/coinj) on days 1, 26, 55, and 90, and blood was taken on days 0 (preimmune serum), 25, 54, 75, and 120. Nonimmunized animals and animals immunized with the vector lacking the insert served as control groups.

Determination of antigen-specific antibody production by ELISA

For ELISA, black 96-well high-bind immunoplates (Fluoro-Nunc; Nunc, Roskilde, Denmark) were coated by overnight incubation at 4°C with purified recombinant Bet v 1a protein (rBet; Biomay, Linz, Austria) at 1 μ g/mL in PBS. Plates were washed with PBS-Tween by using the AW1 automatic ELISA-plate washing device (Anthos Labtec, Salzburg, Austria) and blocked with PBS containing 3% BSA for 2 hours 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 into subsequent columns. For the determination of IgE antibodies, IgG antibodies have been removed by batch incubation with recombinant protein G (Pierce, Rockford, Ill) coupled to Affigel-10 (Biorad, Germany). Preimmune sera served as negative controls. The plates were incubated for 1 hour at 37°C and then washed. Horseradish peroxidase-conjugated detection antibody was added in PBS and incubated for 1 hour at 37°C. For IgG1 and IgG2a detection, goat anti-mouse IgG1 or IgG2a (Pharmingen) was used at 1:2000 dilution in PBS. Readings were referenced to commercial isotype standards, which were mouse IgG1 and IgG2a (Pharmingen).

For total IgG detection, goat-anti mouse IgG(H+L) (BioRad, Germany) and for IgE detection, rat-anti-mouse IgE (Pharmingen), were used in the same way as for the subclass antibodies. The luminometric assay was developed with Luminol (BM chemiluminescence substrate, Boehringer-Mannheim) diluted 1:1 in H₂O. Chemiluminescence (photon counts/second) was determined with 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 with the multiple antibody sandwich principle, according to the protocol supplied by the manufacturer (Genzyme, Cambridge, Mass). 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 by using the standard curves.

Proliferation assay of isolated spleen cells

Spleens were minced in PBS, pH 7.2, and aggregated cells were sedimented for 10 minutes. The supernatants containing a monodisperse cell suspension were washed 3 times in PBS and then resuspended in culture medium (DMEM) supplemented with streptomycin (100 μ g/mL), penicillin (100 U/mL), and 1% heat-inactivat-

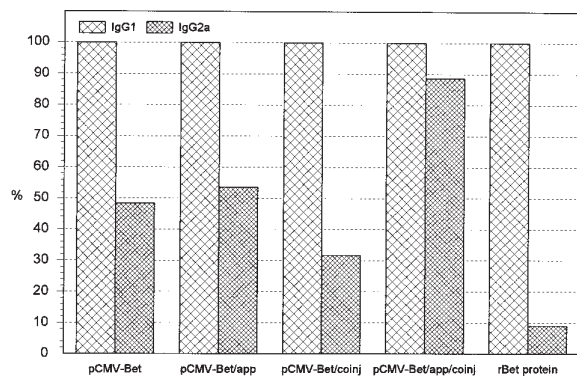


FIG 3. ELISA of IgG1 and IgG2a responses after 3 intraperitoneal injections of rBet v 1a, 3 intradermal injections of pCMV-Bet, and pCMV-Bet modifications. IgG1 values were set to 100% to indicate the ratio between the 2 subclasses. Coinjection of CpG-ODN with pCMV-Bet/app induced the highest proportion of IgG2a, whereas immunization with rBet v 1a protein revealed the highest ratio of IgG1:IgG2a (>1:10).

ed FCS. Cells (1×10^6 /well) were cultured with rBet v 1a (20 μ g/mL) in 96-well, flat-bottom, tissue culture plates for 52 hours at 37°C, 95% relative humidity, 7.5% CO₂. Cells were pulsed with 25 μ Ci/mL [³H]-thymidine (Amersham, Buckinghamshire, UK) for an additional 20 hours and then harvested with a cell harvester (Skatron, Lier, Norway). [³H]-Thymidine incorporation was measured in a liquid scintillation counter (Beckman Coulter).

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical significance was assessed by using an unpaired Student's *t* test.

RESULTS

Antibody responses in pCMV-Bet-treated animals

Mice were injected intraperitoneally with recombinant Bet v 1a protein or intradermally with plasmid DNA encoding the Bet v 1a gene, and IgG, IgG1, IgG2a, and IgE were measured by ELISA. The primary humoral response after DNA-based immunization was weak; however, the reaction could be boosted by 2 additional injections to a level similar to that after protein injection (Fig 2, A). IgE antibodies were detectable only in the group immunized with recombinant Bet v 1a protein (Fig 2, B).

Influence of CpG motifs on antibody responses

To test the influence of CpG motifs on the immune response against Bet v 1a, 2 approaches were used. First, 4 different CpG sequence motifs were appended to the Bet v 1a gene between the 3' end and the stop codon. This construct was designated pCMV-Bet/app. Concerning their effect on stimulating APCs, these 4 CpG motifs are described as "strong CpG motifs" with a 5'-A and a 3'-T flanking the CpG (CpG1 = AACGTT, CpG2 = AACGTC, CpG3 = GACGTT, and CpG4 = GACGTC). In the second approach, coinjection of the CpG-ODN GCTAGACGTTAGCGT²⁴ (CG underlined) with the

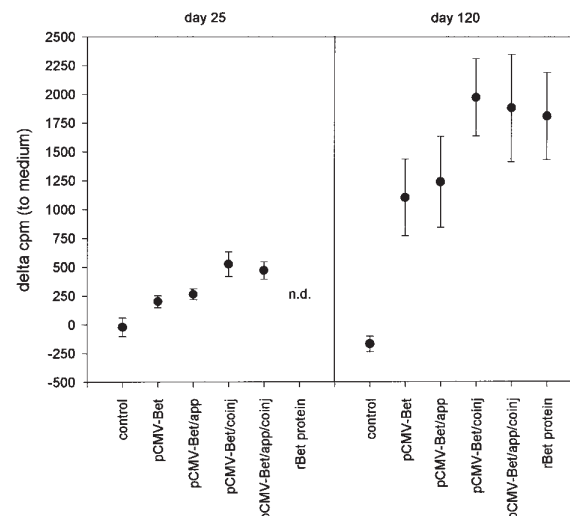


FIG 4. Allergen-specific proliferation of isolated spleen cells. Spleen cells of control and immunized animals were cultured for 52 hours with rBet v 1a and pulsed with 25 μ Ci/mL [³H]-thymidine for 20 hours. Values are expressed in δ cpm (medium), mean $\pm$ SEM. Proliferation of spleen cells revealed an allergen-specific stimulation starting from 3 weeks after the first injection of plasmid DNA. Three further injections increased the proliferative responses. The proliferative response after immunization with the recombinant allergen did not differ from that induced by DNA-based immunization. *n.d.*, Not determined.

pCMV-Bet and pCMV-Bet/app DNA constructs was performed, and the 2 groups of animals were designated pCMV-Bet/coinj and pCMV-Bet/app/coinj, respectively. As shown in Fig 2, A, both appendage of CpGs and/or coinjection of CpGs significantly reduced the IgG antibody response against Bet v 1a ($P < .05$).

IgG2a antibodies were detected in all experimental groups after the first boost (Fig 3). A significantly higher ratio of IgG2a antibodies was only seen in the group pCMV-Bet/app/coinj; immunization with protein induced the highest ratio of IgG1:IgG2a.

In animals treated with the empty pCMV vector, no antibody responses could be detected. Also, coinjection of pCMV-Bet with the non-CpG-ODN sequence GCTAGAGCTTAGGCT in which the CpG motifs were converted to GpC (inverted CG underlined), removing the immune stimulatory effect, did not alter the response when compared with pCMV-Bet alone (data not shown). These results clearly demonstrate the specificity of the antibody responses after immunization with plasmid DNA encoding the Bet v 1a allergen and the inhibitory effect of CpG motifs on IgG responses.

Proliferative responses in spleen cells and cytokine profiles in sera

Despite the lack of antibody response after the first injection of plasmid DNA, all groups showed reactions concerning proliferative responses and serum cytokine levels. Proliferation of spleen cells (Fig 4) revealed an allergen-specific stimulation starting from 3 weeks after the first injection. Three further injections increased the

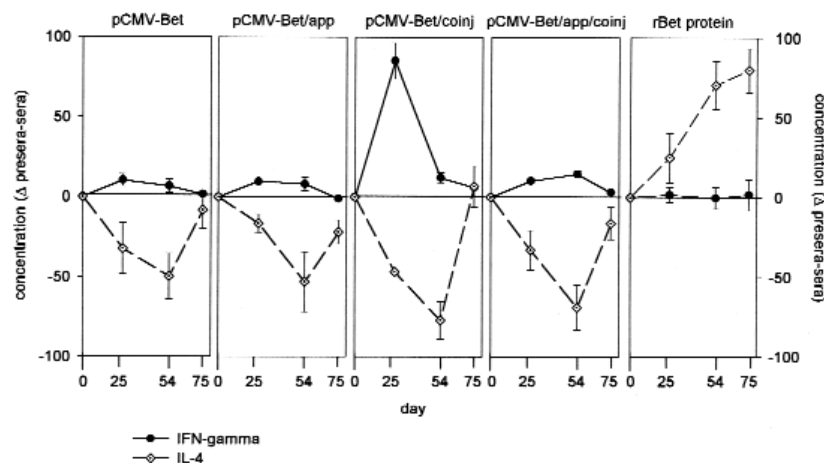


FIG 5. ELISA of IFN- γ and IL-4 in serum of animals ($n = 6$) immunized with pCMV-Bet, pCMV-Bet/app, pCMV-Bet/coinj with 50 μ g CpG-ODN, pCMV-Bet/app/coinj with 50 μ g CpG-ODN, and rBet v 1a. Preserum values were subtracted from experimental values. Data are expressed as mean $\pm$ SEM. IFN- γ was increased after the first and second injection ($P < .05$), and IL-4 decreased ($P < .05$) in all groups injected with plasmid DNA. Coinjection of CpG-ODN with pCMV-Bet revealed the strongest primary response with the most pronounced T_{H1} -type reaction. Immunization with the recombinant allergen induced a clear T_{H2} -type response indicated by a striking increase of IL-4.

proliferative responses. As compared with nonimmunized animals, all experimental groups at both time points showed a significant increase of proliferation with rBet v 1a ($P < .05$) without a significant difference between them. Nonrelated antigens, such as myoglobin and ovalbumin, induced no proliferation, indicating the specific nature of the response (data not shown). The proliferative response of animals immunized with recombinant Bet v 1a did not significantly differ from that of animals immunized with plasmid DNA.

The early onset of cellular reactions was also reflected by changes in the serum levels of IFN- γ and IL-4 observed 25 days after the first injection of plasmid DNA. IFN- γ production was increased ($P < .05$), and IL-4 was strikingly downregulated ($P < .05$) in all groups (Fig 5). This IL-4 suppression was particularly noticeable during the first boost performed 4 weeks after the first injection. After a second boost, the serum levels of both cytokines were normalized, and only a slight decrease of IL-4 compared with the corresponding values in preimmune sera was observed. The highest IFN- γ levels in sera were detected after the first injection of pCMV-Bet/coinj. In contrast to these results, immunization with Bet v 1a protein elicited a predominant T_{H2} -type response with an increased IL-4 production, which did not normalize after the second boost.

Cytokine levels in supernatants of stimulated spleen cells

In general, immunotherapy by DNA-based immunization, like conventional immunotherapy, may also require multiple injections. To determine whether the induced T_{H1} response remained stable under repeated immunizations over a prolonged period, at day 120 (3 weeks after the third booster injection) an allergen-specific proliferation assay was performed by using isolated spleen cells

and rBet v 1a as described above. IFN- γ and IL-4 levels were determined in the supernatant of stimulated spleen cells. IFN- γ levels were increased in all groups by using CpG motifs either appended or coinjected ($P < .05$). IL-4 was strikingly suppressed in all groups ($P < .05$) without any significant difference between them, indicating a clear T_{H1} -type response in all animals. Only mice immunized with pCMV-Bet alone showed no increase in the levels of IFN- γ . Nevertheless, the ratio between IFN- γ and IL-4 for the pCMV-Bet-treated animals also indicated a T_{H1} -type response. The same immunization schedule performed with recombinant Bet v 1a protein resulted in a clear T_{H2} -type proliferative response with a significant increase of IL-4 (Fig 6).

DISCUSSION

Specific immunotherapy (SIT) of Type I allergy is an efficient treatment for patients with pollinosis, and numerous controlled clinical trials have demonstrated the efficacy of this form of therapy.^{23,24} However, as allergens are injected into a sensitized organism, the occurrence of typical allergic symptoms is frequent. Because of these unwanted side effects, therapeutic doses can sometimes not be reached, and SIT has to be considered a potentially dangerous therapy, which in some cases is not successful. Recently, evidence accumulated that SIT influenced the allergen-specific T-helper cell responses.²⁵⁻²⁸ From these studies it can be concluded that a modulation of the allergen-specific immune reactivity from a T_{H2} -orientated to a T_{H1} -biased response is the operating mechanism leading to clinical success. On the basis of these findings, several strategies of inducing T_{H1} -like immune responses to allergens have been established.^{17,18,29}

The recently described features of DNA-based immunization offer several aspects for a new type of allergen-specific immunotherapy. Thus far no side effects, such as

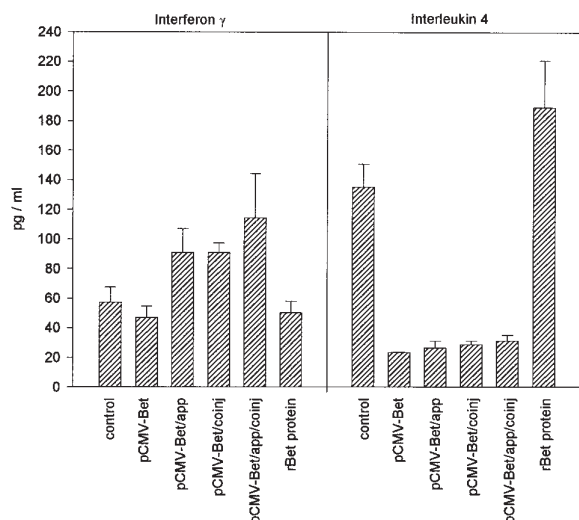


FIG 6. ELISA of IFN- γ and IL-4 in supernatants of proliferation cultures 30 days after 4 monthly injections. Data are expressed as mean $\pm$ SEM. IL-4 suppression was highly significant in all groups ($P < .001$) immunized with plasmid DNA. The IFN- γ increase was detected only in the groups injected with plasmid DNA coinjected with CpG motifs ($P < .05$). An increase of IL-4 was only detectable in the protein immunization group. *Control*, Cytokine expression of spleen cells from nonimmunized animals stimulated with rBet v 1a.

inflammatory responses or anaphylactic reactions, have been detected on immunization with plasmid DNA, which may be due to the minimal concentration of antigen (only nanograms of proteins are translated at the injection site) compared with the doses used for conventional immunotherapy.¹ Furthermore, DNA can be highly purified and thus contain no contaminations, which recombinant proteins may frequently exhibit.

The Bet v 1a protein is known to be a strong allergen, inducing high levels of specific IgE antibodies in about 80% of the individuals allergic to birch pollen. Therefore this molecule seems to be a proper candidate for evaluation of the potential of DNA-based immunization with respect to future strategies of allergy treatment. In our experiments the course of the humoral immune reaction demonstrated that multiple injections of plasmid DNA were necessary to induce a humoral response with high antibody titers against Bet v 1a. In spite of a weak humoral response after the first injection, the animals showed a strong cellular response of a clear T_{H1} -type reaction, as indicated by an increase of IFN- γ and a reduction of IL-4 levels in the serum. Immunization with the recombinant allergen, however, induced a clearly predominated T_{H2} -type response with an early onset of IgG, a characteristic ratio of IgG1:IgG2a, production of IgE, and enhanced IL-4 expression.

The use of CpG motifs (both appended to the Bet v 1 coding region or coinjected as CpG-ODN) did not enhance the humoral reaction to Bet v 1, but a suppressive effect was observed in both cases. These results are in contrast to previous studies dealing with the effects of immunostimulatory DNA sequences on gene immunization.³⁰ CpG motifs are known to activate IFN- γ pathways, which change the intracellular processing by means of low mole-

cular mass polypeptides and transporter associated with antigen processing.^{31,32} Because we used the Bet v 1a gene without a signal sequence that can circumvent the cytosolic degradation of the translated proteins, the CpG-induced altered processing may have reduced the amount of native protein that is a necessary prerequisite for antibody production.

As can be seen in Fig 5, the cytokine serum levels of all animal groups after DNA-based immunization normalized after a third injection, yet still with a slight tendency to a T_{H1} -type reaction. This effect could not be seen after repeated immunization with the recombinant allergen; increasing amounts of IL-4 were still detectable after a third injection. The normalization of the serum levels of cytokines can be interpreted in a positive sense because prolonged changes of systemic cytokine levels may even be harmful. To verify whether the T_{H1} -type reaction remained stable over a longer period of time after multiple injections of plasmid DNA, animals received a fourth injection on day 90, and spleen cells were assayed on day 120. The cytokine profile of the culture supernatants clearly confirmed a still ongoing T_{H1} response. IL-4 was drastically reduced in all groups, and increased levels of IFN- γ were detected, except in the group immunized with plasmid DNA encoding Bet v 1a without any additional CpG sequences. These animals showed no increase in IFN- γ compared with nonimmunized controls. Nevertheless, the ratio of IFN- γ to IL-4 still pointed to a T_{H1} -type response.

No major variations were observed in the distribution of IgG1 and IgG2a subclasses during the whole immunization schedule. Injection of the recombinant allergen induced a characteristic T_{H2} -type subclass response. After DNA-based immunization, both subclasses were detectable with a ratio of 2-3:1 for IgG1. Only the group immunized with pCMV-Bet/app/coinj displayed higher IgG2a levels compared with the other groups. Considering a ratio of IgG1 to IgG2a greater than 10 as an indicator for a typical T_{H2} -type response after genetic immunization,¹² the subclass distribution also indicates a T_{H1} -type response. However, it should be pointed out that the expression of subclasses (without measuring the key cytokines) cannot be seen as a reliable indication of either a T_{H1} - or a T_{H2} -type reaction.³³ Therefore the IgG subclass distribution together with the cytokine profiles (in serum and supernatants of proliferation cultures) provide strong evidence for a T_{H1} response after the immunization with the Bet v 1 gene. Furthermore, in contrast to the immunization with the rBet v 1a protein, no IgE antibodies could be detected after DNA-based immunization in any of the animals at any time during the experiments.³⁴ Similar findings were also reported after DNA immunization of mice with a plasmid construct containing Der p 5, an important house dust mite allergen,¹⁸ and with plasmid DNA encoding the latex allergen Hev b 5.³⁵

In conclusion, our data clearly demonstrate the possibility to induce a strong T_{H1} -type response against the clinically relevant allergen Bet v 1a by a DNA-based immunization and a supportive role of CpG motifs concerning the cellular responses. It remains to be estab-

lished whether an immunization with the Bet v 1 gene can prevent already established atopic immune responses and downregulate an ongoing T_{H2} response.

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