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Strategies for the Development of Safe and Effective DNA Vaccines for Allergy Treatment

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Abstract:

During the past ten years, a great number of studies have demonstrated that injection of plasmid DNA coding for certain genes results in the induction of humoral and cellular immune responses against the respective gene product. The features of DNA vaccines enable a broad range of applications, including the induction of protective immunity against viral, bacterial, and parasitic infections, and open up new perspectives for the treatment of cancer. Furthermore, based on their Th1-promoting properties, DNA vaccines also turned out to balance Th2-mediated immune reactions, a quality which renders them a promising alternative for immunotherapy against allergy. Their unique immunological properties offer new possibilities for the development of vaccines, which do not cause anaphylactic side effects, a major drawback of specific immunotherapy (SIT). In this review, we present approaches to avoid the translation of native allergenic determinants, thus preventing release of allergy mediators stimulated by crosslinking of pre-existing or vaccine-induced IgE antibodies on mast cells. Three approaches are described, which fulfill these requirements: (i) cutting the allergen gene into overlapping fragments, which lack any antigenic determinant of the native allergen, but display the original repertoire of T cell epitopes, (ii) using hypoallergenic derivatives or (iii) fusing the allergen with ubiquitin, thus fragmenting the antigen and destroying its native structure.

The presented experiments demonstrate that DNA vaccines are suitable to balance an allergic response in a protective as well as a therapeutic experimental design, thus demonstrating their potential for allergy treatment.

In addition to conventional plasmid DNA vaccines, aspects and perspectives of replicon-based DNA vaccines will be discussed.

*presenting author

Key words: DNA vaccines, DNA immunization, allergy, plasmid DNA, CpG motifs, immunomodulation, ubiquitination, codon usage, leader sequence, hypoallergenic derivatives

Introduction

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

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

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

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

The downstream events involved in the activation of T cells require the presentation of antigen by professional antigen-presenting cells (APCs) in the context of certain co-stimulatory signals. The absence of appropriate co-stimuli results in anergy or activation-induced cell death by apoptosis (8). A number of studies have demonstrated that of all cell types dendritic cells (DCs) are most effective in recognizing and responding to various "danger" signals (9-11). In general, such signals result from tissue and/or cell damage, but also molecules of bacterial origin such as lipopolysaccharide (LPS) and even bacterial DNA itself can act as "danger" signals. These DNA sequences, also designated as CpG motifs, contain unmethylated CpG dinucleotides flanked by two

5'-purines and a thymine to produce

However, this method does not completely solve the problem of the immune system's inability to translate of protein into a result from protein release, whereas release of protein from cell activation and lack crucial co-stimulatory signals should result in the induction of a lowing DNA in the skin as intradermal injection of DNA induced immune response. Acquisition of a cross-priming effect is related by muscle injection.

One of the major problems is to co-deliver in the same way thus offering unique strategies have succeeded in immunization, such as using adjuvant sequences, costimulatory proteins, and various possibilities, either for both vaccination

DNA immunization

The prevalence of immune dysregulation in autoimmune immunotherapy is either inducing or inhibiting. Regarding the type of immunization

This aspect of antigen presentation in BALB/c mice with IgG2a antibody but no IL-4 or Th2-type response

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5'-purines and two 3'-pyrimidines and were shown to stimulate the innate immune system to produce type-1 helper T lymphocyte (Th1)-promoting cytokines (12,13).

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

One of the major advantages of DNA immunization undoubtedly is the possibility to co-deliver information together with the antigen-coding sequence on the plasmid, thus offering unique opportunities for vaccine development. Recently, several strategies have successfully been used to modulate the immune response after DNA immunization, such as: (i) modification of the vector by inserting or deleting various regulatory sequences, (ii) co-delivery of genes or adjuvant molecules with regulatory and/or stimulatory properties, and (iii) different methods and sites of gene delivery. These possibilities, either as single approaches or in combinations, opened up new perspectives for both vaccine development as well as basic immunological research (3-5).

DNA immunization and allergy

The prevalence for IgE-mediated allergic diseases appears intimately linked to a dysregulation in the balance of Th1 and Th2 lymphocytes (24-26). Current immunotherapeutic approaches for the treatment of allergic diseases generally aim at either inducing tolerance, or redirecting the immune response towards a Th1-type reaction. Regarding the latter, DNA immunization has yielded promising results as a novel type of immunotherapy.

This aspect was first addressed with a DNA vaccine encoding the widely used model antigen *Escherichia coli* β -galactosidase (7). Intradermal DNA immunization of BALB/c mice elicited a typical Th1-biased humoral immune response with elevated IgG2a antibody levels. CD4⁺ T cells isolated from immunized animals secreted IFN- γ , but no IL-4 or IL-5. In contrast, immunization with protein stimulated a predominant Th2-type response with high IgG1 levels, IgE production, and IL-4 and IL-5 expression

in a lymphoproliferation assay. Injection of the DNA vaccine prior to protein immunization prevented IgE formation and, moreover, DNA application following protein immunization elicited a therapeutic effect.

At the same time, the protective anti-allergic effect of DNA vaccines was demonstrated for the clinically relevant dust mite allergen Der p 5 (27). These experiments were performed with rats and also resulted in prevention of IgE synthesis. Furthermore, pre-immunization with the DNA vaccine reduced histamine release in bronchoalveolar fluids and airway hyperresponsiveness after challenge with aerosolized allergen. Transfer experiments indicated an important role of CD8⁺ T cells in anti-allergic effects.

During the past years, a number of clinically relevant allergens have been employed for DNA vaccine studies. We characterized the immune responses after DNA immunization with the major allergen of birch pollen Bet v 1a (28) and demonstrated immunomodulation with CpG motifs and hypoallergenic isoforms of Bet v 1 (29). DNA immunization has also been shown to reduce anaphylaxis after oral gene delivery of chitosan-DNA nanoparticles encoding the dominant peanut allergen Ara h 2 (30).

Several recent publications confirmed the protective and/or therapeutic potential of DNA vaccines with constructs encoding beta-Lactoglobulin (31), Der p 1 and 2 (32), and Der f 11 (33).

Beyond the additional application of CpG motifs, either co-injected or appended to the coding sequence (28), immunomodulation of DNA vaccines was performed with allergen genes fused to IL-18, a cytokine which indirectly acts as Th1 stimulator (34,35).

In contrast to the protective effect, which is reliable under different experimental conditions, the therapeutic effect of DNA vaccines seems to depend on the immunization protocol (31), a fact, which may also account for the antigen-independent anti-allergic properties of DNA vaccines (36). Within a narrow time window following DNA immunization, a strong systemic Th1 effect due to the CpG motifs of plasmid DNA can obviously lead to non-specific suppression of Th2 responses.

Since cDNAs of many important allergens covering the epitope complexity of most relevant allergen sources are already on hand, the prerequisites for construction and clinical evaluation of DNA-based specific allergy vaccines are available (37). However, before DNA-based allergy vaccination can be taken into clinical application, two concerns need to be addressed: (i) The danger of autoimmune responses caused by wide distribution of the transcript (38) and excessive Th1 immune activation induced by DNA vaccination, and (ii) anaphylactic side effects as known from specific immunotherapy (SIT), which are caused by preexisting and/or newly "therapy-induced" IgE antibodies.

The former may be overcome by strictly allergen-specific vaccination protocols, whereas the latter needs new approaches to avoid anaphylaxis during the course of immunotherapy. Both aspects require the development of optimized DNA vaccines with respect to immunogenicity and safety.

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Optimizing the immunogenicity of allergen-specific DNA vaccines

The anti-allergic effect of DNA vaccines depends on the recruitment of Th1 cells and immunogenicity is a necessary prerequisite for DNA-based desensitization approaches. Vector modification, immunomodulation, and co-stimulation are promising attempts to optimize the expression level, the form of the antigen, and the immunostimulatory "danger" signals, which all together are of crucial importance for a strong immune response.

Recoding overcomes the barrier of heterologous codon usage

The expression level of DNA vaccines, which is a decisive parameter for their immunogenicity, is mainly influenced by features of the vector itself. Moreover, suboptimal codon usage of transfected cells concerning heterologous genes (which is the case with DNA vaccines encoding plant allergen genes and mammalian cells as the vaccine target) can severely diminish protein expression.

This can be demonstrated with a DNA vaccine encoding the major mugwort allergen Art v 1. Constructs encoding Art v 1 revealed no or only marginal immune responses. Immunogenicity of the Art v 1 gene vaccine could not be enhanced by approved immune modulations such as the addition of an eukaryotic leader sequence (39), co-injection of a GM-CSF encoding plasmid as adjuvant (40) or cationic liposome-mediated i.d. gene delivery (41). We therefore investigated whether the codon usage of this plant sequence was responsible for the observed effect.

Analysis of the wild-type sequence of Art v 1 revealed that the codon usage frequencies of this gene are quite different from those prevalent in humans. For example two Ala, one Gln, one Ile, two Pro, two Ser and one Thr (Table 1) of Art v 1 are encoded by triplets, which are almost never used in human genes (42). The remaining amino acids also display a codon bias different from those in human genes, though not as drastic.

For recoding the Art v 1 gene (gb:AF502559), the majority of codons was modified (72.4 %). No deviations from strict usage of optimized codons were allowed (Table 1). The human optimized Art v 1 shows a high GC content (71%) and a distinct codon bias for guanosine and cytosine at the third codon position (86.9% G or C at the third codon position) compared to the wild type Art v 1 gene (53% GC and only 35.5 % G or C at the third codon position).

To analyze the *in vitro* expression of both Art v 1 genes, the vector construct with the synthetic gene and the plasmid encoding wild type Art v 1 were used for transfection of B16 mouse melanoma cells. The co-transfected expression plasmid pCMV-β, coding for the reporter gene beta-galactosidase served as an internal standard.

Compared to the wild type gene, the expression level of the codon-optimized Art gene was about 180-fold higher. Only trace amounts of Art v 1 after transfection with the wild type construct could be detected.

Table 1. Recoding of the Art v 1 gene.

Amino Acid	Art v 1 Codons	n for Art v 1	Human codon frequency/1000	Optimized Codon	Human codon frequency/1000 of the optimized codon
Ala	GCT	7	18.6	GCC	28.6
	GCA	2	16.0		
	GCC	1	28.6		
	GCG	2	7.6		
Arg	AGA	1	11.5	CGG	11.9
Asn	AAC	1	19.5	AAC	19.5
Asp	GAT	3	22.3	GAC	26.0
	GAC	3	26.0		
Cys	TGT	5	9.9	TGC	12.2
	TGC	3	12.2		
Gln	CAA	1	11.8	CAG	34.6
Glu	GAA	3	29.1	GAG	40.8
	GAG	2	40.8		
Gly	GGT	7	10.8	GGC	22.9
	GGA	2	16.3		
	GGC	5	22.9		
His	CAT	1	10.4	CAC	14.9
	CAC	2	14.9		
Ile	ATA	1	7.1	ATC	21.5
Leu	TTG	1	12.5	CTG	40.2
Lys	AAA	5	24.0	AAG	32.9
	AAG	7	32.9		
Met	ATG	1	22.3	ATG	22.3
Phe	TTT	2	16.9	TTC	20.4
Pro	CCT	12	17.3	CCC	20.0
	CCG	2	7.0		
	CCA	5	16.7		
	CCC	2	20.0		
Ser	TCA	3	11.7	AGC	19.3
	TCG	2	4.5		
	TCC	2	17.4		
	TCT	3	14.6		
	AGT	1	11.9		
	AGC	1	19.3		
Thr	ACA	2	14.8	ACC	19.2
	ACT	1	12.8		
	ACG	1	6.2		
Trp	TGG	1	12.8	TGG	12.8
Tyr	TAT	1	12.0	TAC	15.6
	TAC	1	15.6		
Val	GTA	1	7.0	GTG	28.9

The codons and their frequency of occurrence of the wild type Art v 1 gene (n for Art v 1 = the number of the respective codons as used in the wild type Art v 1) are compared with the codon frequencies of highly expressed human genes (human codon frequency per thousand). The codon usage data for human proteins are derived from published results (40). The codon-optimized Art v 1 gene always represents the optimal codon concerning the human codon usage (expressed for all optimized codons in the rightmost column).

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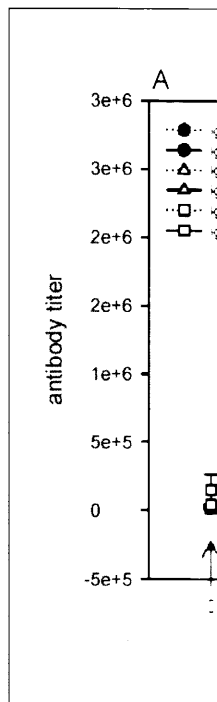


Fig. 1. Art v 1-specific antibody titer in BALB/c mice (n=6) immunized with pCMV-wtArt (filled circles) or pCMV-OptArt (open triangles). The antibody titer for IgG, IgG1 and IgG2a (filled squares, open squares, and open circles respectively) were measured in panel B) are means $\pm$ SEM.

Attachment of secreted antigen

Considering the fact that the translated antigen is secreted into the endoplasmic reticulum (ER) results in the antigen being presented by MHC class II proteins. This process is essential for several antigen-presenting cells to many cell surface receptors. The antigen is then released as soluble molecules, which can be recognized by antibodies without any secreted antigen. The antigen is then released as soluble molecules, which can be recognized by antibodies without any secreted antigen. The antigen is then released as soluble molecules, which can be recognized by antibodies without any secreted antigen.

Figure 1 demonstrates that genetic immunization with the wild type Art v 1 gene induced neither antibodies against the purified natural Art v 1 nor against the recombinant molecule. However, recoding of Art v 1 led to a strong immune response after two booster injections with the typical Th1-biased antibody profile of i.d. genetic immunization (high titers of IgG2a but no IgE antibodies, therefore the latter are not included in the figure). Furthermore, genetic immunization induced antibodies, which obviously recognized both the purified natural as well as the recombinant Art v 1 to the same extent.

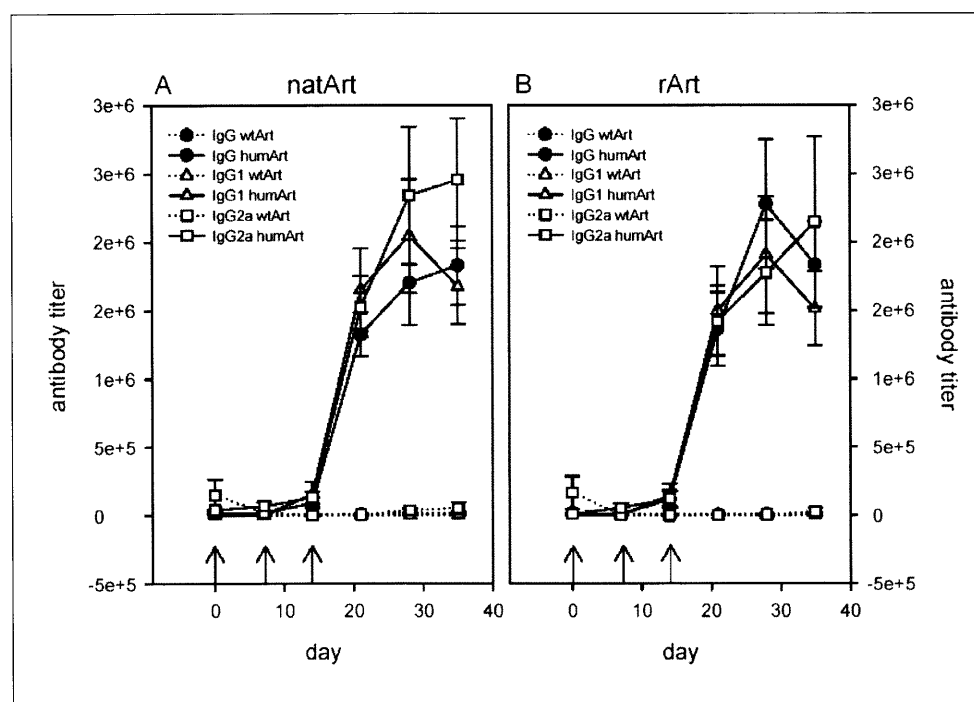


Fig. 1. Art v 1-specific antibody responses with a recoded DNA vaccine.

BALB/c mice ($n=6$) were immunized intradermally with a DNA vaccine encoding the Art v 1 wild type gene (pCMV-wtArt) or the synthetic "humanized" gene (pCMV-humArt), three times in weekly intervals. Total IgG, IgG1 and IgG2a titres against natural purified Art v 1 (natArt, panel A) or recombinant Art v 1 (rArt, panel B) were measured on day 0, 7, 14, 21, 28, 35 and 42. Immunizations are indicated by arrows, values are means $\pm$ SEM.

Attachment of secretion signals improves the immunogenicity of DNA vaccines

Considering that antigen presentation is crucial for immunogenicity, the form of the translated antigen plays an important role. Targeting proteins to the endoplasmic reticulum (ER) results in enhanced secretion, increased amounts of antigen available for MHC class II presentation and improved immunogenicity. This has been demonstrated for several antigens from infectious organisms and cancer (reviewed by (5)). In contrast to many cell surface molecules of pathogens, most allergens are small and highly soluble molecules, which to some extent may be passively released from transfected cells without any secretion signals. This could be the reason why most allergen DNA vaccines induce humoral immune responses although they are not secreted.

Nevertheless, insertion of a leader sequence targeting the antigen to the ER additionally improves the immunogenicity. A DNA vaccine encoding the major birch pollen allergen Bet v 1a together with the human tissue plasminogen activator (hTPA) leader sequence stimulated a five-fold antibody level compared to the non-secreted form (three immunizations in weekly intervals, luminometric ELISA, $7375 \pm 2140 \times 10^3$ relative light units/sec (RLU) with hTPA leader, $1471 \pm 612 \times 10^3$ RLU without hTPA, $p < 0.02$).

Co-delivery of CpG motifs increases the Th1 immunity of DNA vaccines

The extent of the Th1-biased reaction can be modulated by additional application of CpG motifs. Co-injection of CpG-ODN along with the plasmid DNA vaccine represents a simple approach to make use of the Th1-stimulatory capacity of CpG motifs (28,29).

CpG motifs can also be added to the gene of interest by appending a CpG-rich sequence, which codes for certain amino acids. We have attached the amino acid sequence Tyr-Asn-Asn-Val-Asn-Val-Asp-Val-Asp-Val to the carboxyterminus of the Bet v 1a gene, which is encoded by a sequence containing four CpG motifs (28,29). In this case, the attachment did not disturb the correct folding of the protein (29).

Both aspects, appending and co-injection, increased the Th1 bias of the immune response with respect to humoral as well as cellular response.

Optimizing the safety of allergen-specific DNA vaccines

To increase the safety of DNA vaccines, the translation of native allergenic determinants must be avoided in order to prevent anaphylactic responses induced by crosslinking of pre-existing or vaccine-induced IgE antibodies on mast cells. However, in order to guarantee the recruitment of allergen-specific Th1 cells, T cell epitopes must not be destroyed. We investigated three approaches, which in theory should fulfill these requirements: (i) cutting the allergen gene into overlapping fragments, which lack any antigenic determinant of the native allergen but display the original repertoire of T cell epitopes, (ii) using hypoallergenic derivatives or (iii) fusing the allergen with ubiquitin, thus fragmenting the antigen and destroying its native structure.

DNA vaccines encoding hypoallergenic derivatives

DNA vaccines containing the complete Bet v 1a wild type cDNA (pCMV-Beta) and, for reduction of potential anaphylactic side effects, vaccines based on cDNAs coding for two hypoallergenic Bet v 1 fragments (pCMV-Bet1/74 and pCMV-Bet75/160) as well as for a hypoallergenic Bet v 1 mutant (pCMV-Betmut) were constructed (43-45). The protective effect of the DNA vaccines was studied in mice that were pretreated by i.d. DNA injections and then sensitized with aluminum hydroxide-adsorbed Bet v 1 protein. The therapeutic efficiency of hypoallergenic derivatives was tested by sensitizing the animals with recombinant allergen followed by treatment with a DNA vaccine encoding the fragments of Bet v 1.

BALB/c mice immunized with recombinant Bet v 1a protein as well as those genetically immunized with a DNA vaccine encoding the entire Bet v 1 molecule served as

positive control (Fig. 2B). Subclass distribution of antibodies was determined by flow cytometry, whereas DNA vaccines encoding IgG1 and IgG2 were determined by ELISA. A delayed onset of allergic reaction to Bet v 1a (Figure 2C) was observed by the DNA vaccine.

To investigate the effect of the DNA vaccine on the allergic reaction, we had any effect on the allergic reaction.

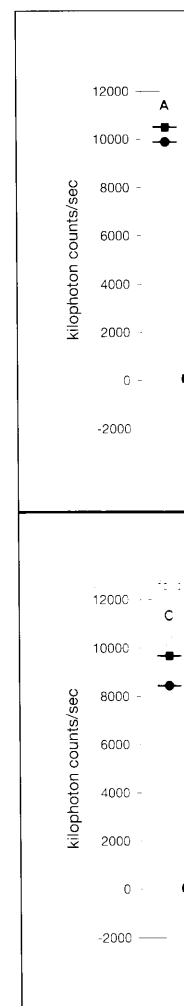


Fig. 2. Bet v 1-specific IgG1 and IgG2 antibody levels in BALB/c mice ($n = 5$) immunized with Bet v 1a (open circles) or a control (filled circles) were determined by flow cytometry. The y-axis represents kilophoton counts/sec on the y-axis.

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positive control. Both produced a strong humoral immune response (Figures 2A and 2B). Subclass distribution after protein immunization was restricted mainly to IgG1, whereas DNA immunization induced expression of high levels of allergen-specific IgG1 and IgG2a antibodies. Genetic immunization with the Bet v 1 mutant elicited a delayed onset and only transient production of both antibody subclasses specific for Bet v 1a (Figure 2C). No antibody response against recombinant Bet v 1a was induced by the DNA vaccine encoding the fragments of the allergen (Figure 2D).

To investigate whether the Th1-type stimulus induced by i.d. genetic immunization had any effect on the subsequent response induced by protein immunization, mice were

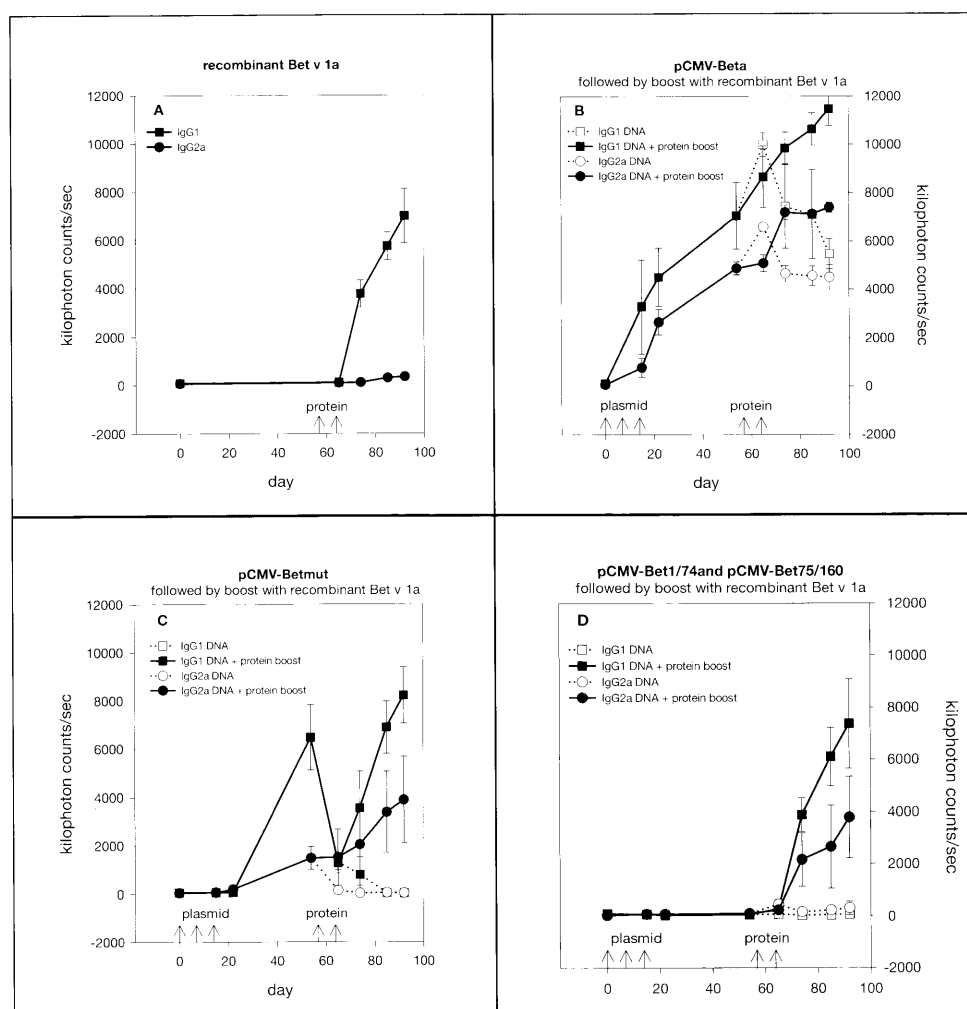


Fig. 2. Bet v 1-specific IgG1 and IgG2a immune responses.

BALB/c mice ($n = 9$) were intradermally immunized with plasmid DNA encoding Bet v 1 and hypoallergenic derivatives, three times in weekly intervals. On days 57 and 64, animals were sensitized s.c. with recombinant Bet v 1a ($n=5$) or left untreated ($n=4$). Bet v 1-specific antibody responses are displayed as kilophoton counts/sec on the y-axis. Immunizations are indicated by arrows, data are expressed as means $\pm$ SEM.

boosted twice with 5 µg recombinant Bet v 1a adsorbed to Al(OH)₃ 6 weeks after the last DNA immunization. In the pCMV-Beta group, a protein boost induced a further increase of IgG1 and IgG2a antibodies. The antibody levels in animals without protein sensitization reached a peak 60 days after the first injection and then declined (Figure 2B).

Both groups, those treated with the Bet v 1 mutant and the Bet v 1a fragment constructs showed a strong induction of IgG1 and IgG2a after the second protein boost (Figure 2C). The influence of DNA-pre-immunization can also be seen by comparing Figures 2A and 2D, which demonstrate that DNA immunization with fragments of Bet v 1a does not induce Bet v 1a specific antibodies but modulates the subsequent protein response by inducing elevated levels of allergen-specific IgG2a antibodies ($p=0.012$ compared to protein immunization). In contrast, protein immunization of a group of animals without DNA pre-immunization primarily induced IgG1 antibodies and only very low levels of IgG2a antibodies (Figure 2A).

Pre-immunization with a DNA vaccine encoding the Bet v 1a unrelated allergen Phl p 5 induced strong induction of IgG1 and IgG2a antibodies against Phl p 5 but revealed no alteration of the Bet v 1a-specific serological Th-type response after sensitization with recombinant Bet v 1a, indicating the antigen-specific nature of the response.

Using a protective experimental design (Figure 3) demonstrated that genetic pre-immunization with pCMV-Beta, pCMV-Betmut and the mixture containing pCMV-Bet1/74 and pCMV-Bet75/160 was able to prevent the induction of IgE antibodies induced by protein sensitization (p ranging between <0.004 and <0.025) (Figure 3A). In contrast, pre-immunization of animals with pCMV-Phl p 5 and subsequent sensitization with rBet v 1a did not prevent the induction of anti-Bet v 1a IgE antibodies (Figure 3B).

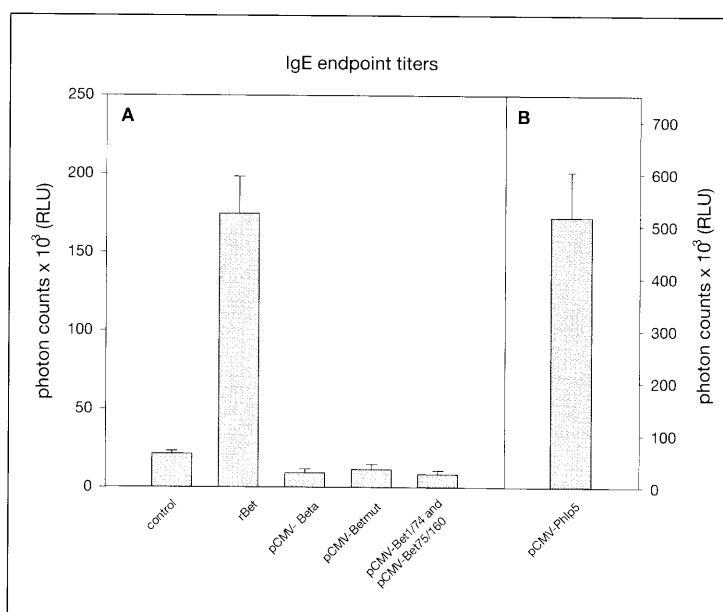


Fig. 3 Levels of Bet v 1a-specific IgE 4 weeks after sensitization (protective approach). Panel A: IgE responses of mice pretreated with plasmid DNA encoding Bet v 1 and hypoallergenic derivatives followed by sensitization with recombinant Bet v 1a. Panel B: IgE response of mice pretreated with plasmid DNA encoding Phl p 5 and sensitized with recombinant Bet v 1a. Control = untreated animals, rBet = sensitization without DNA pre-immunization

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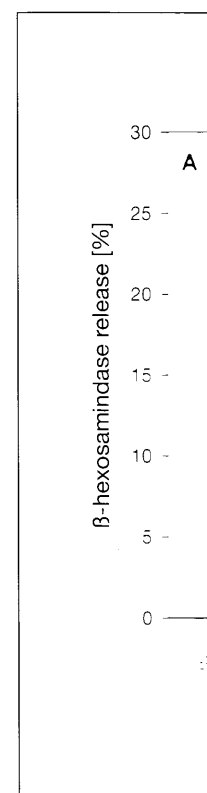


Fig. 4 Rat basophil Allergen-specific IgE. RBL-2H3 cells were sensitized with Bet v 1a. Panel A: Release of histamine from sensitized cells. Panel B: Basophil degranulation with pCMV-Phl p 5

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In order to determine the extent to which genetic immunization can prevent immediate type reactions, an effector cell test was used. The measurement of allergen-specific degranulation of rat basophil leukemia cells (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 birch pollen extract (Figure 4). Sera from Bet v 1 protein-sensitized mice induced Bet v 1-specific basophil degranulation (Figure 4A). In contrast, sera from mice pretreated with Bet v 1-specific genetic immunization did not induce Bet v 1-specific basophil degranulation regardless of a subsequent Bet v 1 protein injection (Figure 4A). Sera from animals genetically pre-immunized with Phl p 5 plasmid DNA and boosted with rBet v 1a contained Bet v 1-specific IgE and induced basophil release ($16.7 \pm 2.3\%$ release; Figure 4B).

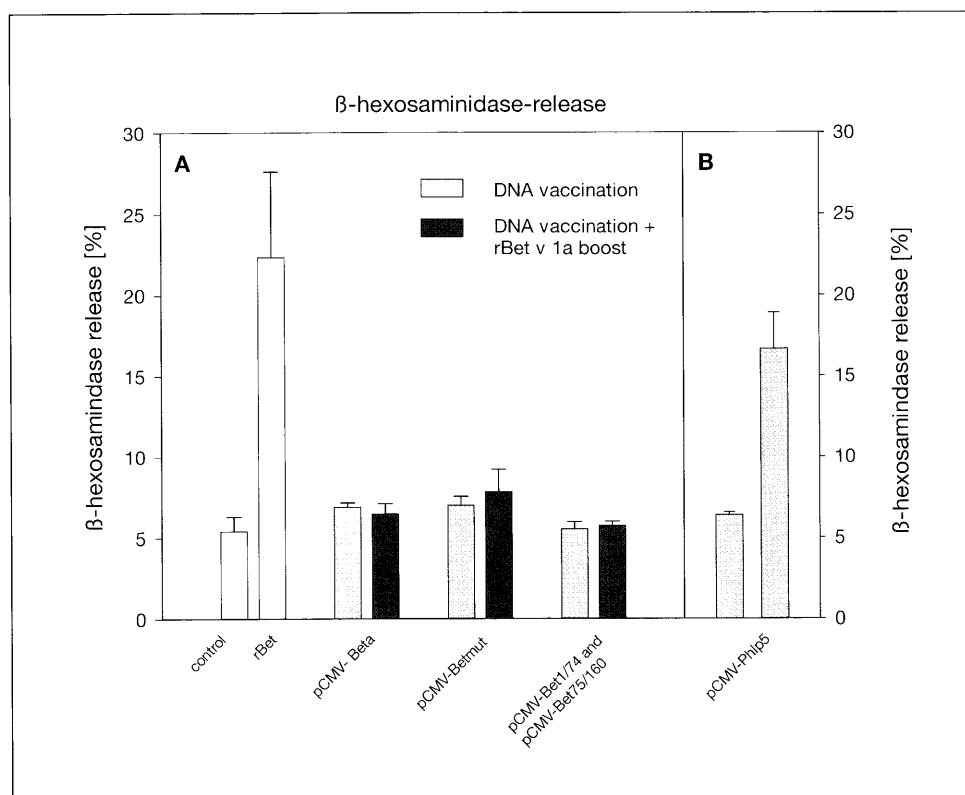
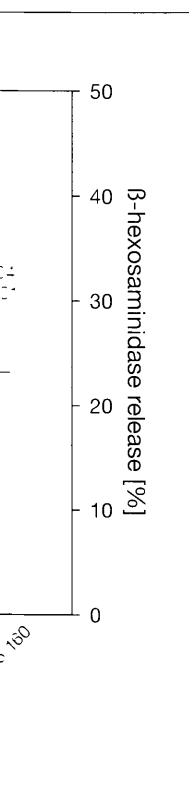


Fig. 4 Rat basophil leukemia (RBL) cell release assay (protective approach).

Allergen-specific degranulation of RBL cells was monitored by β -hexosaminidase release assay. RBL-2H3 cells were passively sensitized with murine sera. Cross-linking of receptor-bound antibodies was performed with $1.5 \mu\text{g/ml}$ birch pollen extract. Results are reported as means $\pm$ SEM for 3-6 mice. Panel A: Release values induced with sera from mice immunized with plasmid DNA alone ($n=4$) or pre-immunized and sensitized with recombinant Bet v 1a ($n=5$). Panel B: Basophil release with sera of mice immunized with pCMV-Phl p 5 plasmid DNA and sensitized with recombinant Bet v 1a ($n=3$).

In order to address the therapeutic capacity of the hypoallergenic DNA vaccine encoding the fragments of Bet v 1a, a Th2-type response was established by sensitizing

5A) in sensitized compared to animals. Measurement of ited a significant



...allergenic frag-
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Lymphoproliferation and cytokine measurement elicited that DNA pre-immunization induced a long-lasting antigen-specific Th1 response associated with IFN- γ production.

The Bet v 1a-specific T-cell response was not restricted to immunization with full-length constructs, but was also detected in mice treated with DNA vaccines encoding the hypoallergens Betmut or both Bet v 1 fragments. No statistically significant differences could be found between groups treated by DNA vaccination.

In order to avoid cross-linking of pre-existing IgE antibodies with the encoded gene product (thus inducing mast cell release), translation can be guided to the proteasome by fusing the gene of interest with ubiquitin. Cytoplasmic proteins are degraded by the ubiquitin-dependent pathway through the action of proteasomes and the resulting peptides are then transported by the Tap1/Tap2 heterodimer into the ER, where they associate with MHC-I molecules and β 2-microglobulin. The resulting complexes are then transported to the cell surface, where they are recognized by CD8⁺ T-cells. Following DNA immunization, only a small percentage of plasmid-encoded proteins becomes ubiquitinated. It has been demonstrated that a stable linkage of ubiquitin to the antigen of interest via a covalent bond efficiently channels the ubiquitinated protein into the polyubiquitination pathway and to the proteasome, thereby shifting the immune response against the encoded antigen towards a Th1-type and simultaneously decreasing or even abolishing antibody reactions while enhancing the cytotoxic response (46-49).

For this purpose we constructed a DNA vaccine encoding a Bet v 1-ubiquitin fusion molecule. Immunization with this vector revealed no antibody production (thus pointing to the supposed degradation of the allergen), but led to 100% suppression of IgE antibodies and IgE-mediated mediator release in a protective experimental design. In the therapeutic situation, IgE production after sensitization ($6668 \pm 2435 \times 10^3$ RLU as measured with luminometric ELISA) was reduced to 11 % by treatment with the DNA vaccine ($782 \pm 188 \times 10^3$ RLU, $p < 0.01$), the IgE-mediated mediator release was reduced from 43.4 % to 28.4 % release ($p < 0.01$). Both experiments demonstrate the functional efficiency of this approach.

The major restriction of genetic i.d. or i.m. needle immunization concerning clinical application is the requirement for large doses of the vaccine. Though there is no evidence for potential harmful events following DNA immunization (50-53), there are concerns about administering milligram amounts of plasmid DNA to humans. Alternative methods for *in vivo* transfection like the gene gun or powderject™ induce high level immune reactions with DNA amounts < 1 µg (54,55). Though these devices are very promising for vaccination against a variety of infectious pathogens, they are

not suitable for the treatment of allergy because of inducing a Th2-biased type of immune response including IgE responses and passive cutaneous anaphylaxis (56).

These hurdles may be overcome by the application of a novel type of DNA vaccines, termed replicase-based genetic vaccines or "DNA-replicons". Immunization with these constructs induces strong humoral and cellular Th1-biased immune responses with nanogram quantities of needle-injected plasmid DNA (57,58).

From self-replicating RNA vaccines to DNA replicon vaccines

The genome of alphaviruses encodes a replicase enzyme used by the virus to efficiently replicate the viral genome inside the host cell cytoplasm (Figure 6). Since this machinery is independent of the host's replication system, it works in a wide variety of cell types and species. Replacement of the genes encoding structural proteins with the sequence of the antigen of interest yields a "self-replicating" RNA vaccine or "replicon". This type of RNA vaccine can effectively compensate for the degradation of RNA occurring *in vivo* as well as *in vitro*. In theory, host cells only need to pick up a single molecule of this self-replicating RNA and still produce sufficient amounts of antigen to induce an immune response. It was previously shown that antigen production in cells transfected with self-replicating RNA (or the replicase-based DNA analogue) is independent of the amount of RNA or DNA used for transfection (57-59).

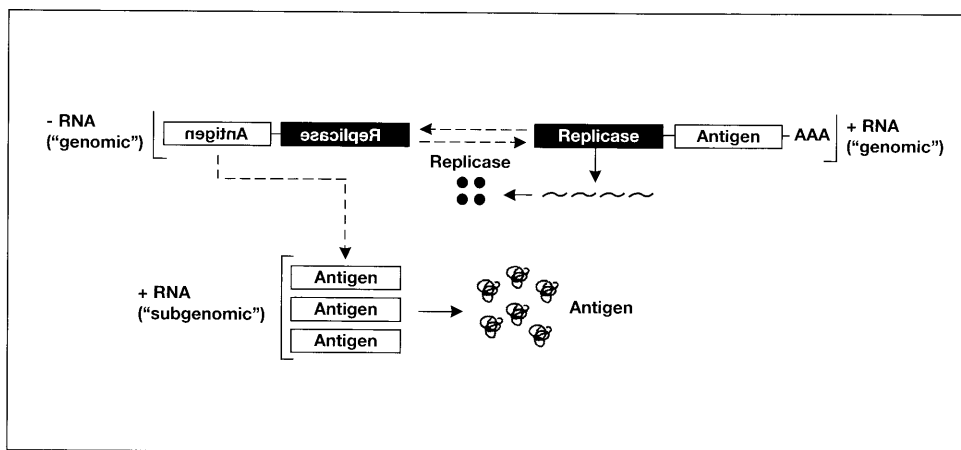


Fig. 6 Principle of self-replicating RNA vaccines.

The first product after translation from self-replicating RNA is the replicase protein, which replicates the RNA-vaccine (or the genomic RNA, in the case of the alphavirus). Negative strand RNA functions as template for both more copies of positive strand "genomic" RNA as well as for "subgenomic" mRNA encoding the antigen (or the capsid proteins, in the case of the alphavirus).

Although RNA vaccines encoding alphaviral replicase can compensate for RNA degradation *in vivo*, RNA stability still remains a problem for these vaccines during *in vitro*-production. To facilitate and simplify vaccine production and handling as well as the storage requirements for vaccines, the sequence of the self-replicating RNA molecule is reverse-transcribed into DNA and cloned into a conventional DNA plasmid (Figure 7). Now, the self-replicating RNA is produced by transfected cells from this plasmid using a conventional promoter (57-59).

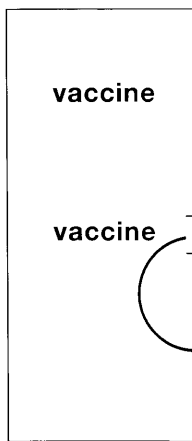


Fig. 7 Conversion of a plasmid DNA replicon into a self-replicating RNA vaccine.

DNA replicons

Similar to the self-replicating RNA vaccines, transfected cells producing the immune response into the chromosome of the organism.

The high efficiency of the triggering power of the encoded antigenic immune response (57,58,60).

DNA replicons

We investigated a construct encoding a plasmid DNA vaccine 100 to 1000-fold Th1-promoting new form of vaccine conventional plasmid 10 µg DNA replicon vaccine induced a Th1-biased response with 1 µg of injected

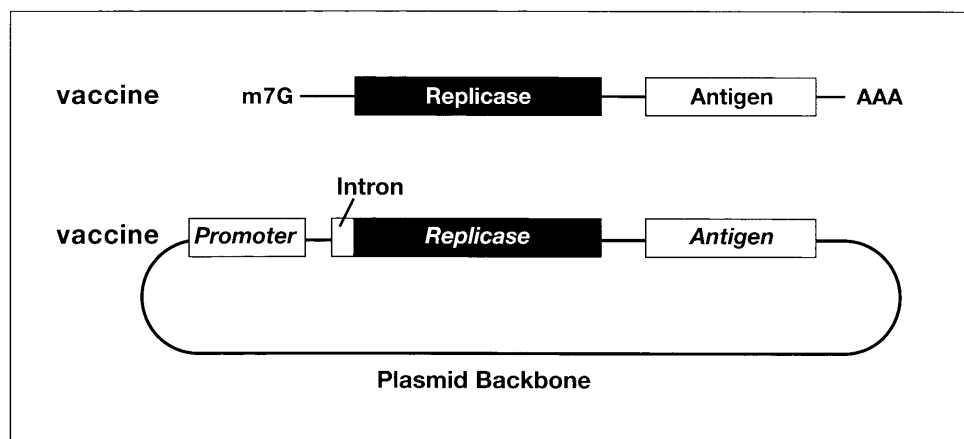


Fig. 7 Conversion of self-replicating RNA vaccines into replicase-based DNA vaccines. "Plasmid DNA replicons" are created by reverse transcription of the RNA sequence into DNA and insertion into a DNA plasmid. Production of the self-replicating RNA is then driven by a promoter on the plasmid (e.g., CMV promoter).

DNA replicons fulfill the requirements for safe and effective vaccines

Similar to infection of cells with alphaviruses, transfection of mammalian cells with self-replicating RNA based on alphaviral replicase causes host cell apoptosis (58,60), probably a mechanism to limit viral spread. In case of "self-replicating" genetic vaccines, transfected cells containing the plasmid may be cleared from the organism during the immune response. This peculiarity of replicon-based vaccines could overcome one of the major criticisms against genetic immunization: the risk of stable integration into the chromosome due to long-time persistence of high amounts of plasmid DNA in the organism.

The high effectiveness of self-replicating RNA vaccines and "DNA replicons" is most likely the result of mimicking a viral infection of transfected host cells, thereby triggering powerful antiviral pathways, which enhance the immune response against the encoded antigen. Therefore, "DNA replicons" induce humoral and cellular immune responses at doses 100 to 1000-fold lower than conventional genetic vaccines (57,58,60).

DNA replicons can induce both, Th1- as well as Th2-biased immune type responses

We investigated the principle suitability of DNA replicons for allergy treatment with a construct encoding the model allergen β -galactosidase. In contrast to conventional plasmid DNA vaccination, the amount of DNA administered with DNA replicons is 100 to 1000-fold lower, which means a drastic reduction of the concentration of the Th1-promoting CpG content. Therefore, one of the crucial questions was, whether this new form of vaccine induced similar types of immune responses compared to the conventional plasmid DNA vaccine. For this purpose, needle immunizations with 1 μ g and 10 μ g DNA replicon vaccine were compared to gene gun immunization with 1 μ g DNA replicon vaccine. The results in Figure 8 demonstrate that needle immunization induced a Th1-biased immune reaction (increased levels of IgG2a antibodies, Panel B) with 1 μ g of injected DNA and was even superior to gene gun immunization (Panel A).

In contrast, 1 μ g of a conventional plasmid DNA vaccine does not induce a measurable immune response or Th1-promoting effect. Therefore, the detected Th1 bias after needle injection of this small amount of replicon-based DNA must be triggered by a different mechanism than the CpG effect induced with high dose DNA vaccination. A reasonable explanation seems to be that DNA replicons mimic a viral infection, thus stimulating Th1-biased serological and cytokine patterns (58,60). Gene gun immunization overruled the Th1-promoting effect (Figure 8B), which confirms recent findings that gene gun immunization stimulates a particular and dominant Th2 signal (61). In agreement with recent data concerning gene gun immunization with DNA vaccines coding for the genes of Bet v 1a, Phl p 2, and β -galactosidase (56) this also argues against the use of gene gun DNA immunization for allergy treatment.

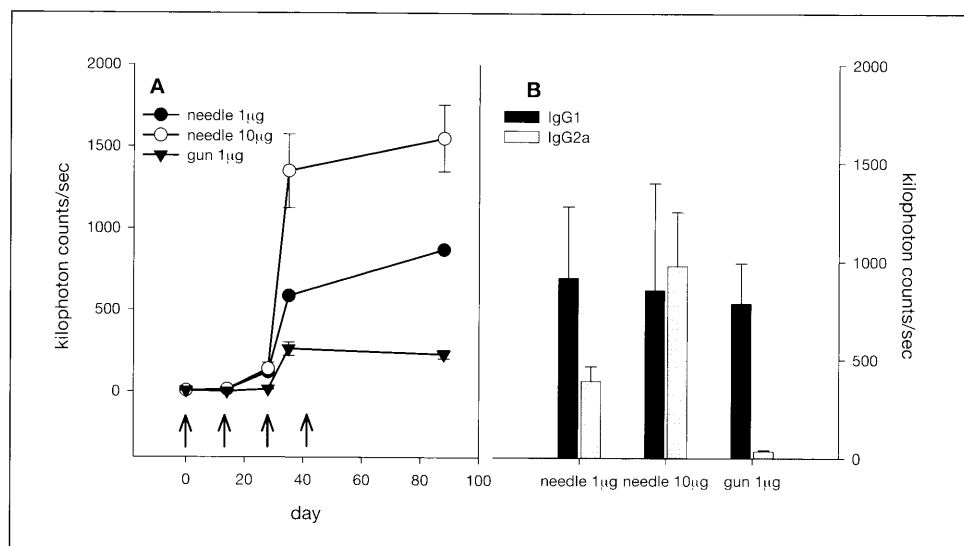


Fig. 8 Immune response types after immunization with plasmid DNA replicons.

BALB/c mice were immunized with plasmid DNA encoding β -galactosidase four times in two week intervals either by i.d. needle immunization (1 μ g or 10 μ g DNA) or by gene gun (1 μ g DNA).

Panel A: Total IgG levels.

Panel B: Subclass distribution at day 90. Specific antibody responses are displayed as kilophoton counts/sec on the y-axis. Immunizations are indicated by arrows, data are expressed as means $\pm$ SEM.

Summing up, the features of DNA replicons render them proper candidates to balance allergic responses in the same efficient way, which has already been demonstrated with conventional plasmid DNA vaccines.

Concluding remarks

The experiments and results described above represent an extract of the enormous modulatory potential of DNA vaccines focused on their application in the field of allergy. Although DNA immunization enables highly sophisticated approaches in vaccine research and development, the necessary prerequisite for the experiments is molecular biological and immunological standard methodology.

With respect to restricted to practical applications (3).

In general, modification of the allergen may be the high priority. In many cases, the allergen is a protein, as was the case of A. fumigatus, which further supports the case of A. fumigatus immunogenicity. DNA vaccines are a construct with the desired, constructed.

With respect to the immunizing approach, the effects due to the immunization on mast cells.

Fragmentation of the allergen approaches. The structure and the function of the allergen.

Ubiquitination of the allergen for knowledge of the allergen and the use of quin vectors.

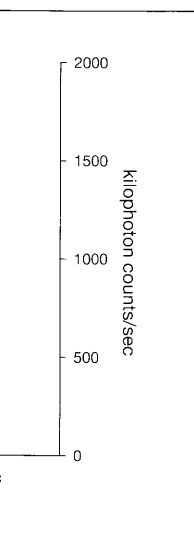
The application of the allergen in allergic effector cells is carefully monitored and the immune response.

Summing up, the application of the allergen in vaccine research and treatment of allergy.

Acknowledgments

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duce a measurable Th1 bias after need- triggered by a dif- A vaccination. A aral infection, thus ne gun immuniza- ns recent findings Th2 signal (61). In th DNA vaccines also argues against



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Discussion

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