



Protective efficiency of dendrosomes as novel nano-sized adjuvants for DNA vaccination against birch pollen allergy

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Abstract

We evaluated the use of a novel gene porter (Den123-a nontoxic self-assembled dendritic spheroidal nanoparticle made of biodegradable monomers), aiming to enhance and improve the desired immune response in protection from allergy. Footpad DNA immunization in Balb/c mice was done three times using the Bet v 1a gene with or without Den123 with 2-week intervals followed by sensitization with rBetv1 (5 µg) in alum twice in a weekly interval. Different doses of pCMV-Betv1 were used (10 µg and 100 µg). The protective role of different formulations was evaluated by measuring the IgG1, IgG2a and IgE antibody production, cytokine release of isolated splenocytes and β-hexosaminidase release from the RBL cells. Higher and increasing ratios of IgG2a/IgG1 were seen in mice which received plasmids in combination with Den123. Den123 and DNA vaccine synergistically enhanced the Interferon gamma released from splenocytes. In the presence of Den123, IgE inhibition was independent of the dose and type of the injected DNA. All DNA-pre-immunized mice demonstrated low basophil degranulation. It is therefore concluded that administration of the DNA entrapped in Den123 nanoparticles results in sustained release of plasmids, Th1/Th2 balanced immune response with promising IgE inhibition. Also higher amounts of DNA contributed to stronger Th1 response. © 2006 Elsevier B.V. All rights reserved.

Keywords: Dendrosome; DNA vaccine; Allergy; Nanoparticle; Bet v 1a; Adjuvant

1. Introduction

The prevalence of diseases such as allergic asthma, atopic dermatitis, and hay fever has increased markedly in the developed world over the past 40 years (Wills-Karp et al., 2001). Conventional allergy

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immunotherapy which consists of administering small but increasing concentrations of allergens over a long period has been used in the treatment of atopic diseases for nearly 100 years. The drawback of this approach is that systemic administration of the active allergens can cause severe IgE-mediated side effects including anaphylactic reactions induced by cross-linking of the pre-existing IgE antibodies on mast cells. Moreover, the injection of high doses of antigen can lead to anaphylaxis during the course of treatment via stimulation of “therapy-induced” IgE antibodies. During the treatment the therapeutic effective doses often cannot be achieved because of the non-standardized extracts or side effects. Specific immunotherapy (SIT) is an inconvenient procedure with the requirement of intensive medical supervision (Bousquet et al., 1998; Stewart and Lockey, 1992; Wolf and Hamilton, 1998). Although the immunotherapy is successful in reducing the clinical symptoms in 60–90% of the cases, few patients are cured (Creticos, 2000). Moreover, despite the long history of SIT, the underlying mechanisms involved are not yet fully understood (Durham et al., 1999).

It has been reported that immunization with an allergen encoding plasmid DNA suppresses not only the IgE formation but also anaphylaxis and allergic inflammation in rodent models. In the initial proof-of-principle experiment, a DNA vaccine encoding the model antigen beta-galactosidase was employed (Raz et al., 1996). 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 corresponding protein stimulated a predominant Th2-type response characterized by high IgG1 levels, IgE production, and IL-4 and IL-5 expression. Injection of the DNA vaccine before protein immunization prevented IgE formation, and moreover, DNA application after protein immunization elicited a therapeutic effect. The anti-allergic effect of DNA vaccines can be clearly attributed to the recruitment of Th1 cells and the establishment of a balancing Th1-biased cytokine milieu which inhibits de novo synthesis of IgE antibodies. A major advantage of nonviral DNA vaccines is their high safety profile. The lack of viral antigens (like with viral vector vaccines) and serious side effects in animals and humans makes them attractive, espe-

cially when multiple immunizations are required. The amount of antigen translated after DNA immunization in the skin is at least three to five orders of magnitude lower than the amount of protein injected during a typical SIT protocol. Furthermore, the high purity of the DNA vaccine avoids the induction of new allergic reactions via cross-reacting impurities, a problem of SIT with allergen extracts. It has been established that the resulting immune response is usually influenced by the route of administration (Gurunathan et al., 2000). The site of administration is another factor that determines the dose of the DNA needed for achieving the protection. Also Th-type reaction is modulated by the site of injection.

Many vaccines require repeated immunizations to assure complete protection. Single-administration vaccine (SAV) formulations automatically avoid such a problem. Polymer systems for vaccine delivery have been studied over the past 20 years and have shown promise as SAVs (Cleland, 1995; Thomasin et al., 1996; Singh et al., 1992; Lee et al., 1997; O Hagan, 1997). Polymers investigated include linear cationic polymers such as polylysines, branched polymers such as polyamidoamines or phosphorous-containing dendrimers, polyethyleneimines which bind electrostatically to the negatively charged DNA forming complexes that can be readily taken up by target cells. Some natural polymers such as chitosan and alginate have also been used. Polycations such as polylysine, polyornithine, chitosans and polyethyleneimines condense the extended structures of plasmids into a tightly packed complex (Sarbolouki et al., 2000). Collagen based materials release plasmid DNA in times ranging from hours to days (Scherer et al., 2002; Samuel et al., 2002). HA-based hydrogels also release the DNA; however, the rate of release can be controlled by the extent of cross-linking (Yun et al., 2004; Kim et al., 2003). Sustained release has been achieved with hydrogels composed of PEG with lactic acid segments, based on the number of degradable lactic acid units (Quick and Anseth, 2003). The release of DNA entrapped in polymers into biological milieu typically occurs through a combination of diffusion and polymer degradation. This type of delivery may enhance gene transfer efficiency by protecting the DNA from degradation and maintaining the vector at effective concentrations, extending the opportunity for internalization and integration. Microspheres and nanospheres

are two forms of vehicles used for gene delivery. Microspheres, with diameters ranging from 2 to 100 μm , are not readily internalized, but retained within the tissue to release DNA (Mathiowitz et al., 1997; Ochiya et al., 1999). Released DNA can transfect cells at the delivery site, with the protein product acting locally or distributed systemically (Bonadio et al., 1999). In contrast, Nanospheres are particles with diameters ranging from approximately 50 to 700 nm (Panyam and Labhasetwar, 2003), consistent with the size of viral and some nonviral vectors. When such nanoparticles internalize they release the DNA intracellularly. Nanoparticles containing encapsulated antigens can be effective as an adjuvant because they have the capability to provide sustained release of the antigen. Also the sub-micron size of nanoparticles offers a distinct advantage over the microparticles in that nanoparticles have in general a relatively higher intracellular uptake compared to microparticles (Panyam and Labhasetwar, 2003). Some polymers and oligosaccharides have been evaluated for administration of allergens or DNA vaccines in allergy prevention or treatment. It has been shown that oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy and shows a substantial reduction in allergen-induced anaphylaxis, IgE, plasma histamine and vascular leakage (Roy et al., 1999). Poly-D,L-lactide-co-glycolide (PLGA) microparticles have been investigated as a vehicle for administration of the olive allergen Ole e 1 and it is found that its molecular integrity and the antigenic properties remain unaltered by the encapsulation processes or polymer degradation (Batanero et al., 2002). Alginic acid oligosaccharides have been shown to be able to suppress Th2 development and IgE production by inducing IL-12 production. The production of IFN- γ induced by the antigen stimulation was upregulated by ALGO in a dose-dependent manner (Yoshida et al., 2004).

It was previously reported that dendrosomes (a family of novel, nontoxic, neutral, biodegradable, covalent or self-assembled, hyperbranched, dendritic, spheroidal nanoparticles) are easy to prepare, inexpensive, highly stable and easy to handle and apply, compared with other existing synthetic vehicles for gene delivery (cationic lipids, liposomes) (Sarbolouki et al., 2000). Gene delivery into human liver (Huh7), kidney (G293, Vero) and endothelial cells as well as other

mammalian cells like Bowes, U-937, Raw, CCRF-CEM, MOLT-4 and K562, reveals that the genes are efficiently expressed and in comparison with other gene porters like Lipofection or bacterial ghosts, dendrosomes do quite well. Cell culture experiments as well as intraperitoneal/intradermal injections of dendrosomes into mice have established their nontoxicity (up to 2.5 mg/kg of animal weight in the latter case) (unpublished data). Dendrosomes have several generations including Den123, Den450 and Den700. In previous studies it has been shown that Den123 is quite capable of safely delivering the HBsAg (Hepatitis B surface antigen) gene into target cells in Balb/C mice and the immune response achieved in mice receiving pCMV-HBS with Den123 intramuscularly are much higher compared to those receiving plasmids with sucrose or PBS (Sarbolouki et al., 2000). Also plasmid/Den123 ratio of 150 was established to be the best ratio in these experiments (Sarbolouki et al., 2000). In another set of experiments it has been shown that using hepatitis C virus (HCV) core pcDNA3 construct in combination with Den123 (a plasmid to Den123 ratio of 150:1) will result in the same immune response as CpG motif does in Balb/C mice (unpublished data, Khatami et al.). Den123 is a self-assembled nanoparticle formed from a fork-shaped amphipathic monomer. Den123 nanoparticles consist of a hydrophobic core surrounded by a fuzzy shell of hydrophilic chains.

In the present study we have investigated the efficacy of Den123 nanoparticles in delivering the DNA vaccine for allergy prevention. We were aimed at investigating the impact of the dose of plasmids administered on the immune response. For this purpose 10 μg of plasmid (10% of the amount of plasmid conventionally administered) was injected for allergy prevention. The Th1 or Th2 adjuvanticity of Den123 was also checked. We also used footpad injection of plasmids as a novel route of DNA vaccination for allergy prevention.

2. Materials and methods

2.1. Mice

Six to eight weeks old female Balb/C mice were obtained from the breeding stock maintained at the Pasteur Institute of Iran. Mice were dealt with according to Pasteur Institute's guidelines for animal care.

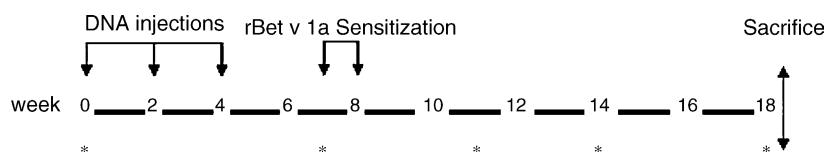


Fig. 1. Experimental setting used for the protective vaccination. Arrows indicate the weeks at which mice received DNA or protein. Asterisks indicate bleedings.

2.2. Experimental design

In order to evaluate the effect of Den123 in protecting mice from Th2 allergic response and to evaluate the impact of the dose of the plasmids administered, and also to address whether the effects of DNA pre-immunization were antigen-specific or derived from Den123 itself, groups of ten mice were used as below. In the first stage of experiments there were six groups: Group I received 100 μg pCMV-Betv1 plus Den123. Group II received 10 μg pCMV-Betv1 plus Den123. Two other groups received 100 (Group III) or 10 μg (Group IV) pCMV-Betv1 in PBS. Group V received 10 μg p-CMV with Den123. The last group (Group VI) received PBS. In the second stage, five mice of each group were sensitized with 5 μg recombinant Bet v 1a(r-Betv1) plus Alum. Remaining mice were injected with sterile PBS as non-sensitized mice (Fig. 1).

2.3. Plasmids

Plasmids pCMV-Betv1 and pCMV were transformed into the *E. coli* DH5 α strain. Large-scale purification of vectors was conducted with Endo Free Plasmid Giga kits (Qiagen) according to the manufacturer's instructions. Plasmid DNA was analyzed by agarose gel electrophoresis, quantified by spectrophotometry (OD 260/OD 280 ratio >1.8). Then plasmids were stored in endotoxin-free H₂O at -20°C .

2.4. Recombinant allergen

The lyophilized rBet v 1a was reconstituted in pyrogen-free water and stored aliquoted at -20°C .

2.5. Dendrosome123

Den123 was synthesized under sterile condition as previously mentioned (Sarbolouki et al., 2000). It was mixed with PBS and left at ambient temperature for

15 min. The suspension formed, was filtered through 0.22 μm filters (Schleicher & Schuell). Final solution (10^{-4} mg ml $^{-1}$) was stored at 4°C .

2.6. Preparation of Plasmid-Den123 nanoparticles and injection

Hundred or 10 μg of plasmids were mixed with needed amounts of Den123 just before injections so that a plasmid to Den123 ratio of 150:1 was prepared for all groups receiving DNA. The mixture was left 5 min at ambient temperature. Final volume for DNA vaccination was 120 μl in sterile PBS which was injected into both footpads of mice (60 μl each). DNA immunizations were performed three times once every 2 weeks (days 0, 14 and 28).

2.7. Preparation of r-Bet v 1a-alum and injection

Mice were sensitized with subcutaneous injection of 5 μg rBetv1 together with 100 μl alum (Serva, Heidelberg, Germany) in a total volume of 170 μl sterile PBS (2–3 sites on the back of shaved mice). Sensitization was performed twice with 1-week interval. The first rBetv1 injection was done three weeks after the last DNA injection.

2.8. Sera collection

Mice were bled from retro-orbital and sera were prepared from the whole blood samples of each mouse by incubating blood samples for 1.5 h at 37°C and then centrifuging for 1 min at 8000 rpm. Sera of each group were pooled and stored at -20°C .

2.9. Enzyme-linked immunosorbent assay

rBet v 1a-specific serum antibody titers were determined by ELISA. Briefly, Costar (NY, USA) 96-well microtiter plates were coated with (100 μl per

well) rBetv1 at $10 \mu\text{g ml}^{-1}$ in PBS and incubated overnight at 4°C . Plates were washed three times with $150 \mu\text{l}$ PBS-0.05% Tween-20 and then blocked with $100 \mu\text{l}$ PBS-1% BSA for 2 h at 37°C . Serum samples were diluted to 1:50 and 1:10 for IgG and IgE, respectively, with PBS-Tween-1% BSA. After washing three times, $100 \mu\text{l}$ of diluted sera was added to each well. Plates were incubated for 2 h at 37°C and then washed. Hundred microliters of biotinylated goat anti-mouse IgG1 (1:600) (Southern Biotechnology Associates, Birmingham, AL, USA), rat anti-mouse IgG2a (1:200) (Pharmingen) and goat anti-mouse IgE (1:25) (Pharmingen) in blocking buffer, were added then incubated for 2 h at 37°C . After washing, $100 \mu\text{l}$ of streptavidin-horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL, USA) was added at 1:4000 in PBS and was incubated at 37°C for 1 h. After washing six times, detection was performed with the substrate *O*-phenylenediamine (OPD, Sigma, USA). Optical density at 490 nm was determined on ELISA reader (Tecan, Austria) with OD range up to 4.0.

2.10. Rat basophil leukemia cell mediator release

RBL-2H3 cells were plated in 96-well tissue culture plates (4×10^4 per well) and incubated for 24 h at 37°C using 7% CO_2 . Passive sensitization was performed by incubation with murine sera (from the 18th week) raised against the birch-pollen allergen Bet v 1a at a final dilution of 1:100 for 2 h. To remove unbound antibodies, the cell layer was washed twice with Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl_2 , 1.8 mM CaCl_2 , 0.4 mM NaH_2PO_4 , 5.6 mM D-glucose, 12 mM NaHCO_3 , 10 mM HEPES and 0.1% BSA, pH 7.2). Cross-linking of the Fc ϵ R-bound IgE and subsequent degranulation of RBL cells was induced by adding $100 \mu\text{l}$ of a $0.3 \mu\text{g ml}^{-1}$ solution of rBetv1 in Tyrode's buffer for 30 min in a humidified atmosphere at 37°C . Supernatants were analyzed for β -hexosaminidase activity by incubation with $80 \mu\text{M}$ 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide (Sigma) in citrate buffer (0.1 M, pH 4.5) for 1 h at 37°C . The reaction was stopped by addition of $100 \mu\text{l}$ glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and fluorescence was measured at $\lambda_{\text{ex}}:360/\lambda_{\text{em}}:465 \text{ nm}$ using a fluorescence microplate

reader (Spectrafluor, Tecan, Austria). Results are reported as percentage of total β -hexosaminidase released after addition of 1% Triton X-100. This assay was done in triplicate.

2.11. In vitro cytokine release

At the end of the experiment three mice of each group were sacrificed and spleens were aseptically removed. Spleens from each individual mouse were homogenized through a tissue grinder and erythrocytes were lysed at room temperature using ACK lysis buffer (NH_4Cl , KHCO_3 , Na_2EDTA). Splenocytes were washed and resuspended in complete RPMI medium (RPMI-1640 supplemented with 5% FCS, 1% L-glutamine, 1% HEPES, 0.1% 2ME, 0.1% Gentamicin). Cells were then seeded at a density of $3\text{--}5 \times 10^5$ cells per well in the presence or absence of rBetv1 ($20 \mu\text{g ml}^{-1}$), and ConA ($5 \mu\text{g ml}^{-1}$) as a positive control in total volume of $200 \mu\text{l}$. The plates were incubated for 4 days at 37°C in 5% CO_2 humidified atmosphere. Supernatants of these wells were taken after 4 days of incubation and stored in -70°C . IFN- γ in supernatants of re-stimulated splenocytes was quantified by sandwich-based ELISA kit (R&D, Minneapolis, MN, USA) according to the manufacturer's instruction manual.

2.12. Statistical analysis

Statistical differences were determined by student's *t*-test using SigmaPlot, version 8 (Jandel Corp., San Rafael, CA, USA). Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Antibody response

The antibody response before sensitization (7 weeks after the first DNA injection) was different regarding its strength and bias among groups which received different amounts of plasmids with or without Den123 (Fig. 2). IgG2a was seen exclusively in mice which received $100 \mu\text{g}$ pCMV-Betv1 alone (Group III) and its level in other groups was low at this time point. It was revealed that during this period higher amounts of DNA resulted in significantly higher IgG1 levels

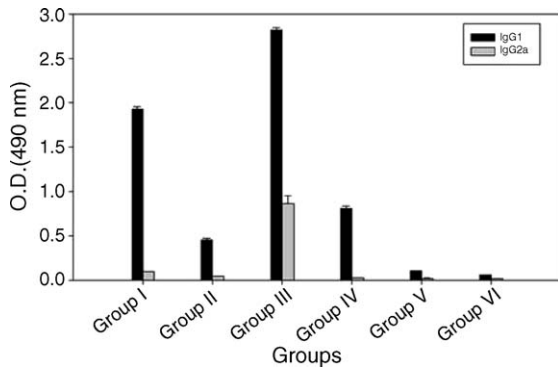


Fig. 2. Bet vl-specific IgG1 and IgG2a responses 7 weeks after the first DNA immunization. Balb/C mice ($n=10$) of different groups were immunized in footpad with: 100 μ g pCMV-Betv1 + Den123 (Group I); 10 μ g pCMV-Betv1 + Den123 (Group II); 100 μ g pCMV-Betv1 (Group III); 10 μ g pCMV-Betv1 (Group IV); 10 μ g pCMV + Den123 (Group V); PBS (Group VI), three times with 2-week intervals. Data are expressed as mean $\pm$ S.E.M.

irrespective of the presence of Den123. Interestingly, IgG1 in animals which received Den123 in combination with pCMV-Betv1 was lower than that of mice injected with DNA alone. IgG1 and IgG2a titers in Groups V (10 μ g pCMV plus Den123) and Group VI (control) were very low. It was shown that IgG1 and IgG2a in non-sensitized mice of Groups I and II reached their highest levels at the 11th week; by contrast their levels in non-sensitized mice of Groups III and IV reached to their highest values at the 7th week and then began to decrease (Fig. 3A and B). It was revealed that rBetv1 administration results in IgG1-restricted antibody response as it was shown in Groups V and VI (Fig. 3C) although IgG2a in Group V reached a high value at week 14 but again decreased. In mice that had received pCMV-Betv1 there was a balance between IgG1 and IgG2a, although the balance depended on DNA dose and formulation. Three weeks after the last sensitization, IgG1 of all pCMV-Betv1 pre-immunized mice increased and remained almost unchanged until the end of the study (Fig. 4A). IgG2a level in all pCMV-Betv1 injected mice increased after protein boost but the largest increase in IgG2a titer occurred in Group I (Fig. 4B). Monitoring IgG2a level until the end of the experiment revealed a consistent increase in IgG2a in Groups I and II which received Den123. After the 11th week IgG2a of Groups III and IV began to decrease and this decrease continued until

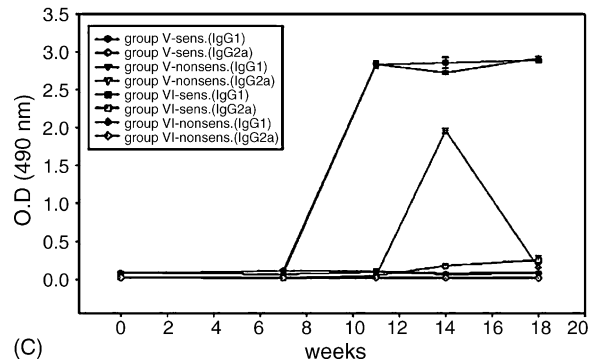
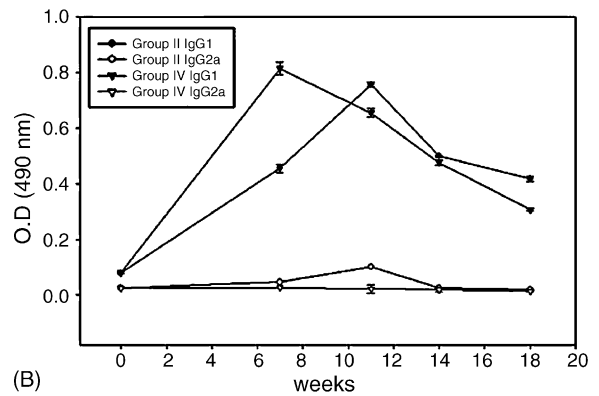
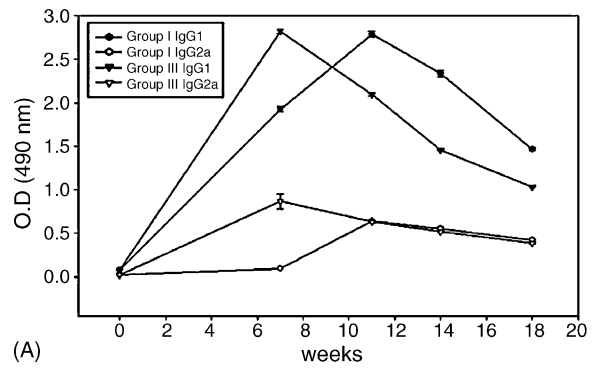


Fig. 3. Bet vl-specific IgG subclasses in sera of: (A) non-sensitized mice of Groups I (100 μ g pCMV-Betv1 + Den123) and III (100 μ g pCMV-Betv1); (B) non-sensitized mice of Groups II (10 μ g pCMV-Betv1 + Den123) and IV (10 μ g pCMV-Betv1) and (C) sensitized and non-sensitized mice of Groups V (10 μ g pCMV + Den123) and VI (PBS). Data are expressed as mean $\pm$ S.E.M.

the 18th week. IgG2a/IgG1 ratio can be regarded as an indicator of Th1/Th2 balance. This ratio changed through the experiment in pCMV-Betv1 injected mice (Fig. 5). In Groups I and II this ratio increased consis-

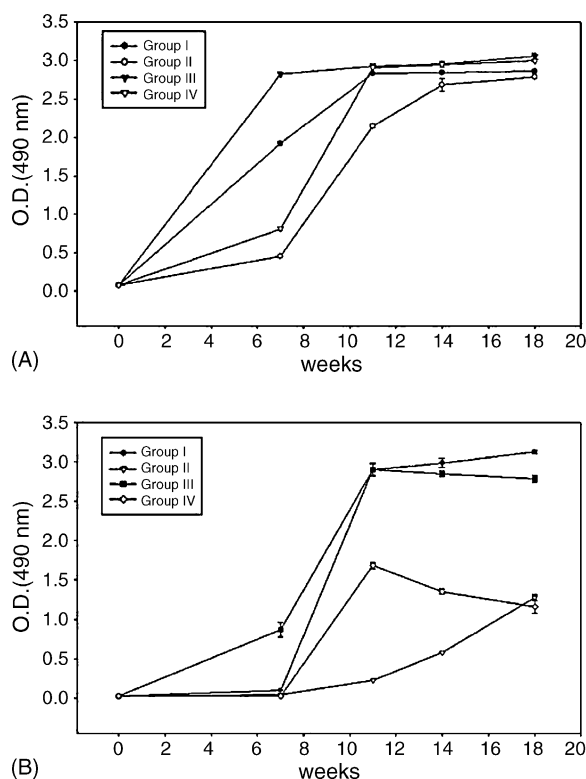


Fig. 4. Betv1-specific IgG1 (A) and IgG2a (B) in sera of pCMV-Betv1 pre-immunized mice (Groups I, II, III and IV) sensitized subcutaneously at the shaved back with 5 µg rBetv1 together with 100 µl Alum at days 49 and 56. Data are expressed as mean $\pm$ S.E.M.

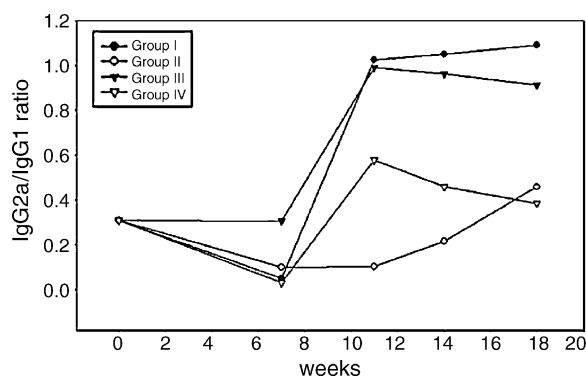


Fig. 5. IgG2a to IgG1 ratio in sera of pCMV-Betv1 pre-immunized mice (Groups I, II, III and IV) sensitized subcutaneously at the shaved back with 5 µg rBetv1 together with 100 µl alum at days 49 and 56.

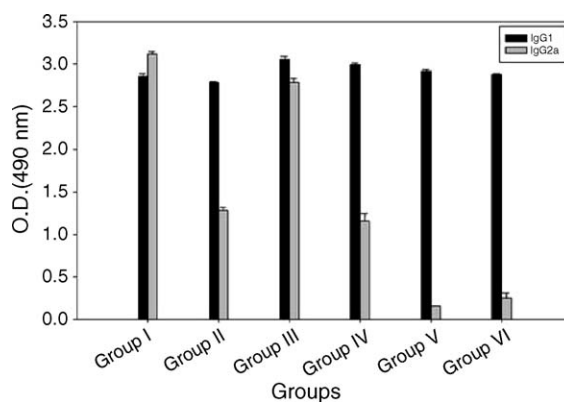
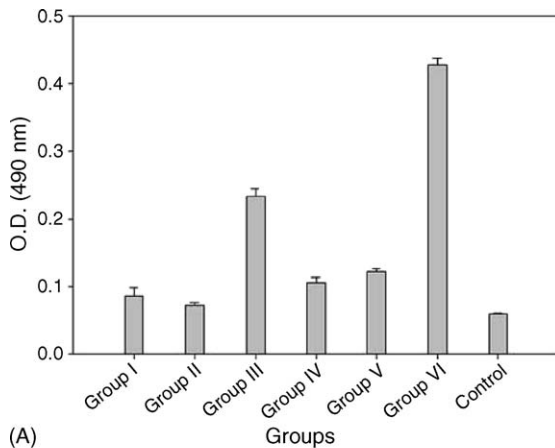


Fig. 6. IgG1 and IgG2a levels in sensitized mice of all experimental groups at the end of experiment (18 weeks after the first DNA immunization). Data are expressed as mean $\pm$ S.E.M.

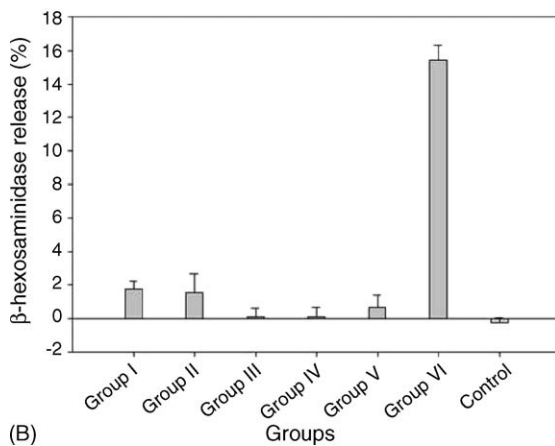
tently until the end of the experiment which indicates the slow release of DNA from Den123; but when DNA was injected alone this ratio reached its highest value at the 11th week and then began to decrease (Groups III and IV). At the end of the experiments IgG2a/IgG1 ratio of Groups III and IV was lower compared to Groups I and II, respectively. It is worth noting that higher amounts of DNA injected contributed to the higher IgG2a/IgG1 ratio regardless of its combination with Den123. Eighteen weeks after the first pCMV-Betv1 administration it was shown that a strong long lasting antibody response (IgG1 and IgG2a) has been induced (Fig. 6). Groups I and III that received 100 µg DNA showed high levels of allergen specific IgG1 and IgG2a antibodies but Groups II and IV had more IgG1 than IgG2a. Specific anti-Betv1 IgE was measured in sera 10 weeks after the last sensitization (Fig. 7A). Among the DNA administered animals, mice of Group III had the highest IgE titer ($P < 0.01$) but their IgE level was lower than Group VI ($P < .004$). IgE levels in Groups I and II were lower than Groups III and IV ($P < 0.01$, $P < 0.05$), respectively. Specific IgE level in Group V was significantly lower than that of Group III ($P < 0.008$) and VI ($P < 0.0009$). All groups had a significant difference with Group VI ($P < 0.004$).

3.2. RBL assay

In order to determine the extent to which DNA immunization by Den123 can prevent immediate type



(A)



(B)

Fig. 7. (A) Bet v 1a-specific IgE levels in sera of sensitized mice of all experimental groups 10 weeks after the last sensitization. (B) Sera from day 126 of sensitized mice of all experimental groups were tested for their anaphylactic activity by measuring the release of β -hexosaminidase. (Control: non-sensitized mice of Group VI.) Data are expressed as mean $\pm$ S.E.M.

reactions, an effector cell test was used. The measurement of allergen-specific degranulation of RBL-2H3 cells passively sensitized with murine sera of the 18th week was performed by the detection of β -hexosaminidase release after cross-linking of receptor bound antibodies with rBetv1. β -Hexosaminidase is an enzyme which is released during degranulation of mast cells and basophils. Therefore, the rat basophil leukemia (RBL) cell release assay provides a functional read-out for IgE-mediated degranulation. Sera from Bet v 1a protein-sensitized mice (Group VI) con-

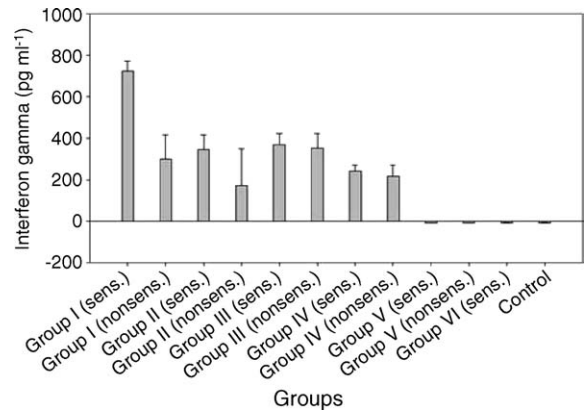


Fig. 8. Secretion of IFN-gamma in supernatants of stimulated spleen cells. Spleen cells of mice ($n = 3$) from all groups were harvested at day 126 and cultured in the presence of 0 (medium) or 20 μ g/ml rBetv1. IFN-gamma levels of rBetv1-stimulated splenocytes (96 h incubation) were measured by Sandwich-ELISA in pg ml^{-1} . Control: non-immunized mice. Data represent mean $\pm$ S.E.M.

tained high levels of Bet v 1-specific IgE (Fig. 7A), and thus induced Bet v 1-specific basophil degranulation (Fig. 7B). β -Hexosaminidase released from Group VI was significantly higher than those of other groups ($P < 0.01$). There was no significant difference among DNA-immunized mice of different groups and the control group.

3.3. Cytokine assay

IFN-gamma level was determined in the supernatants of re-stimulated spleen cells (Fig. 8). Group I had the highest IFN-gamma compared to all other groups ($P < 0.04$). There was no significant difference among other pCMV-Betv1 pre-immunized groups (II, III and IV). rBetv1 boosting did not have significant effect on IFN-gamma expression in DNA pre-immunized mice except the fact that in Group I that the difference between sensitized and non-sensitized mice was significant ($P < 0.05$). No IFN-gamma was detected in V, VI and non-immunized control groups.

Table 1 shows three factors that can be accounted for Th1-type response indicators. IgE inhibition values were calculated by comparison to IgE level of mice which did not receive any DNA and were sensitized by rBetv1 plus alum (Group VI).

Table 1
IgE inhibition, IFN-gamma production and IgG2a/IgG1 ratio at the end of the experiment (day 126)

Treatment	IgE inhibition (%)	IFN-gamma (pg ml ⁻¹)	IgG2a/IgG1 (ratio)
100 µg pCMV-Betv1 + Den123 (Group I)	80	721.6 ± 49.2 ^b	1.10
10 µg pCMV-Betv1 + Den123 (Group II)	83	345.33 ± 69.2	0.46 ^c
100 µg pCMV-Betv1 (Group III)	45 ^a	370.2 ± 52.2	0.91
10 µg pCMV-Betv1 (Group IV)	75	241.0 ± 32.2	0.38

Mice were immunized as mentioned in the text. On day 126 (18 weeks after the first DNA immunization) sera were obtained and mice were sacrificed. Splenocytes of three mice from each group were used for IFN-gamma measurement. IgE inhibition values were calculated by comparison to IgE level of mice which did not receive any DNA and were sensitized by rBetv1 plus alum (Group VI). Data reported are mean ± S.E.M.

^a Indicates that IgE inhibition in mice which received 100 µg pCMV-Betv1 alone was significantly lower than other DNA-immunized groups.

^b Indicates that IFN-gamma production in mice which received 100 µg pCMV-Betv1 with Den 123 was significantly higher than other DNA-immunized groups.

^c Indicates that IgG2a/IgG1 ratio in Group II is lower than that of Group III; whereas IFN-gamma production of splenocytes of this group is comparable to Group III and also IgE level in sera of Group II is significantly lower than Group III.

4. Discussion

Currently it is widely accepted that Th1/Th2 type immune response and duration of high antibody titer after DNA vaccination are determined by some factors including: dose of DNA administered, plasmid expression system, route of administration (intranasal, oral, intramuscular, intradermal, subcutaneous, rectal), site of injection (back, ear, footpad), method of administration (needle, needle-free), the schedule of injection, the adjuvant and the vehicle for administration (liposome, cationic polymers, dendrimer . . .) (Dean et al., 2003; Gurunathan et al., 2000; Cui et al., 2003; Ito et al., 2003). It is logical that for each antigen all factors must be checked to finally find the best combination of these parameters for DNA vaccination. There is much interest in developing new delivery systems for DNA vaccination because of the degradation of DNA by enzymes in vivo. Recently it was shown that Lipofectin (a liposome) can induce higher IgG1 and IgG2a when combined with pDfl (DNA vaccine for Der f 11, a mite allergen) but failed to down-regulate IgE antibody response (Peng et al., 2004). Nano- and microparticles containing entrapped or adsorbed antigens have been investigated as vaccine adjuvant alternatives to the currently used alum with an objective to develop better vaccines adjuvant and minimize the frequency of immunizations. It was shown that PLGA microspheres alone protect most mice against anaphylaxis through the transient induction of the IgG2a and on production of IL-10. These markers were not affected by the presence of DNA which argued for dominant immunomod-

ulatory properties of microspheres (Jilek et al., 2004). It was shown that immunization with the pDNA-coated nanoparticles (ID jet injection and SC injection) led to an enhanced and Th1/Th2 balanced cytokine release profile compared to immunization with naked pDNA alone (Cui et al., 2003).

Using lower amounts of plasmids may be preferred for two reasons: first, the use of DNA as a preventive or therapeutic agent has raised some safety concerns, though its application in vaccination has shown no certain danger. Secondly, from a biotechnological point of view the total costs for preparing a vaccination dose that contains lower amounts of plasmid is of paramount importance and preference.

The results thus described strongly demonstrate that selection of a new site for administration of the vaccine based on Den123 nanoparticles significantly improved the immune response needed for protection from allergy. The Bet v 1a protein induces high level of IgE antibodies in about 80% of the individuals allergic to birch pollen. Therefore, Bet v 1a seems to be a proper candidate for assessing the potential of this new delivery strategy in protection against allergy (Haiti et al., 1999).

Considering IgG1 and IgG2a levels during the experiment in different groups it is revealed that Den123 has resulted in slow release of DNA. After three DNA injections and before sensitization only Group III had noticeable IgG2a indicating the roles of plasmid dose and Den123 since Group I which received the same amount of DNA (100 µg) in combination with Den123 showed very low levels of IgG2a. Also

10 µg plasmid injected into Group IV did not make any difference in IgG2a level. Three weeks after protein boost IgG2a and IgG1 of all sensitized groups increased but the highest IgG2a increase was seen in Group I ($P < 0.0007$). In this group IgG1 and IgG2a were approximately the same. After the 11th week IgG1 level of all groups remained nearly unchanged until the 18th week. IgG2a levels of groups receiving Den123 (I and II) increased consistently. This pattern of IgG2a/IgG1 ratio can be inferred as controlled release of DNA from Den123 nanoparticles. It seems that a portion of the plasmids given to Groups III and IV are degraded by nuclease and the rest is immediately exposed to cells and the earlier response in Group III compared to other groups points to this mechanism. Lower dose of plasmids (10 µg) can be regarded as the cause for the lower response in Group IV. But plasmids when combined with Den123 are supposedly entrapped within nanoparticles which reside intracellularly. The degradation of Den123 and plasmid diffusion result in slow release of plasmids. Interestingly, comparison between sensitized and non-sensitized mice of Group I showed that IgG1 and IgG2a of non-sensitized mice reached their highest levels at the 11th week but after this time both IgGs began to decrease. These results clearly show an 11-week delay needed for reaching high IgG2a levels when DNA is combined with Den123. This is in line with data from experiments done by oral administration of chitosan nanoparticles containing Der p 1 DNA vaccine (Chew et al., 2003). In those experiments the kinetics of antibody production revealed that an 8 weeks delay was needed to induce noticeable serum antibodies. Similar effects have also been observed with a malaria-transmission-blocking antigen (TBV25H), for which three immunizations with a conventional alum formulation did not yield complete protection from infection but a single immunization with TBV25H PLGA microspheres resulted in complete protection as the pulse of antigen happened 40 days after injection (Cleland et al., 1997). In the present study, IgG2a of non-sensitized mice of Group I was significantly lower than sensitized ones. Also protein boost could increase IgG2a drastically in Group I. These data points to the presence of Th1 cytokine milieu after pCMV-Betv1/Den123 administration and that the exposure to rBetv1 contributes to the expansion of the existing IgG2a producing memory B cells. Such a pattern has been reported in studies done by Langer et

al. in which the largest increase in antibody response, after challenging the mice with β -Gal protein, was seen in POE-2 (molecularly engineered polyorthoester microsphere) administered mice (Wang et al., 2004). Also IgG1 at the 11th week was the same for both sensitized and non-sensitized mice of Group I. As mentioned above IgG1 of sensitized mice of Group I did not change after the 11th week which suggests that the rBetv1 boost could not increase IgG1 levels more than that induced by DNA but could maintain the IgG1 level. The steady level of IgG1 in sensitized mice of Group I is advantageous because of the need for a balance between Th1 and Th2 reactions. Simultaneous induction of Th1 and Th2 is needed for parasitic defense and also prevention from autoimmune diseases. So protein boost is inevitable in achieving protection. Comparison between sensitized mice of Group I and mice of Group VI which had an IgG1-restricted response indicates the Th1 adjuvanticity properties of Den123 nanoparticles. At the end of the experiment the ratio of IgG2a to IgG1 was higher in groups receiving 100 µg pCMV-Betv1 regardless of the presence of Den123 nanoparticles but in Groups II and IV which received 10 µg pCMV-Betv1, IgG2a levels were at most 46% of IgG1. This clearly shows the effect of DNA dose on immune response bias.

The rBetv1 specific IgE antibody level was measured 10 weeks after the last sensitization in all groups. Mice of Group III had the highest IgE level among DNA immunized groups. IgE level of Group III was significantly lower than that of Group VI. The effect of Den123 on IgE inhibition is clearly seen when one compares the level of the specific IgE between groups receiving DNA alone and those that have received the same amount of DNA in the form of DNA/Den123. The IgE titer of Group I was significantly lower than Group III and so was IgE of Group II compared to Group IV. Interestingly Den123 has shown dominant IgE-inhibitory effect on pCMV-Betv1 since IgE level in Group V was lower than Group III. This is promising, as mice of Group V were only given empty vector (pCMV) in combination with Den123 and had IgE levels comparable to mice of Group I which were given both Den123 and pCMV-Betv1. Also in the presence of Den123, the effect of plasmid dose on IgE inhibition was lost since Groups I and II had comparable IgE inhibition but Groups III and IV had significantly different IgE levels. It was expected that IgE level of Group V

must be comparable to that of Group VI but the difference was significant indicating the IgE-inhibitory effect of Den123.

RBL assay was done with sera of day 126. Results of this assay confirm Th1 adjuvanticity of Den123 since all DNA-pre-immunized mice, even mice of Group V showed very low β -hexosaminidase release. It was expected that β -hexosaminidase release of Groups V and VI will be similar but, like IgE results, sera of pCMV-immunized mice showed significantly lower release compared to rBet v 1a-sensitized mice. Considering the results of IgE and RBL assay, it can be concluded that Den123 can inhibit IgE production and its consequent basophil degranulation nonspecifically, since Group V which received only 10 μ g empty vector (pCMV) with Den123 could inhibit basophil degranulation like 100 μ g pCMV-Betv1 (with or without Den123).

In addition to IgE-inhibitory mechanism for protection from allergy, the recruitment of antigen-specific Th1 cells and production of a Th1 cytokine milieu are also considered as other anti-allergic mechanisms. These two mechanisms are clearly reflected in the data presented here. To verify whether IgG2a/IgG1 ratios at the end of experiment are indicators of Th1 or Th2 reaction, IFN- γ level in supernatant of restimulated splenocytes was measured. Results clearly confirmed a still ongoing Th1 response in all pCMV-Betv1 pre-immunized groups but IFN- γ level in Group I was much higher than other groups. Groups II, III and IV had comparable IFN- γ levels but no IFN- γ was detected in Groups V, VI and control. High IFN- γ level in Group I proposes a synergistic effect of plasmid dose and Den123 nanoparticles since Group II which received just 10 μ g pCMV-Betv1 plus Den123 had an IFN- γ amount similar to Group III (100 μ g pCMV-Betv1 alone). The IFN- γ level in non-sensitized mice of Group I was comparable to mice of Group III which were sensitized. The higher IFN- γ level in sensitized mice of Group I compared to non-sensitized ones of this group points out to a high population of Th1 cells specific for Bet v 1a which were recruited after administration of pCMV-Betv1 plus Den123. Taking into account data of cytokine and proliferation assays (data not shown), the Th1 adjuvanticity of Den123 nanoparticles containing 100 μ g pCMV-Betv1 is clearly shown and it indicates that there is a large number of Th1

cells in Group I which after rBetv1 sensitization expand largely and contribute to the high IFN- γ in sensitized mice of Group I. Considering a ratio of IgG2a to IgG1 greater than 0.1 as an indicator of a typical Th1-type response after DNA vaccination (Feltquate et al., 1997), the IgG subclass distribution showed that all pCMV-Betv1 pre-immunized mice, have a Th1-type response, although the ratio was dependent on the dose of plasmid injected and also on the presence of Den123. This conclusion was confirmed by IFN- γ expression and proliferation data of mice splenocytes. Higher doses of DNA and Den123 synergistically increased the level of IFN- γ . Moreover, the IgE-inhibitory effect of nanoparticles was clearly confirmed even when Den123 was given with empty vector. In Group V the IgE-inhibitory effect of Den123 was accompanied by Th2 response as IgG1 of this group was much higher than IgG2a and no IFN- γ was detected in splenocytes of this group. Transient induction of the Th1-type antibody IgG2a at 14th week was followed by IgE inhibition in mice which received only empty vector combined with Den123 nanoparticles. There was no synergistic effect of DNA associated with nanoparticles in down-regulating the IgE. But the cytokine assay showed synergistic effect of DNA combined with Den123 in up-regulating IFN- γ . Jilek et al. reported similar observations using PLGA microspheres but they did not observe synergistic effect in IFN- γ up-regulation (Jilek et al., 2004). Despite the inhibitory effect of pCMV-Betv1 on IgE production in different DNA pre-immunized groups, concomitant high levels of IgG1 was seen. It was previously found that anaphylactic IgG1 accompanies low IgE level in some mouse strains but not with others (Li et al., 1999). No simple relationship between the amount of DNA and the Th-type of the immune response has been proposed so far (Weiss et al., 2002). However, in the present study we observed a lower IgG2a/IgG1 ratio, a lower IgG total (data not shown) and a higher IgE inhibition in mice which received 10 μ g plasmid compared to 100 μ g-DNA injected mice whether combined with Den123 or not. When DNA was injected alone, IFN- γ level in mice which received 100 μ g DNA was higher than those injected with 10 μ g, but the difference was insignificant ($P > 0.05$). In the presence of Den123 nanoparticles, the IFN- γ level was significantly higher in 100 μ g injected-mice.

Thus, the data reported is the first of its kind in achieving a prolonged inhibition of an allergic reaction to a strong allergen that is modulated by footpad injection of a neutral dendrimeric nanoparticle, Den-drosome123. This approach does not abrogate Th2 response (which is involved in protection against parasites and also prevents autoimmune reaction) completely. The exact mechanisms of IgG2a enhancement from these DNA-entrapped nanoparticles remain to be elucidated. However, it may be speculated that an enhancement in the uptake of DNA-nanoparticle complex by antigen presenting cells (APCs) in the skin may have contributed to this effect. APCs, especially DCs present in the skin, are well equipped for capturing particles (Banchereau and Steinman, 1998). Following their intracellular uptake and endo-lysosomal escape, nanoparticles could release the encapsulated DNA at a sustained rate resulting in sustained gene expression (Panyam et al., 2002). The mechanism of IgE inhibition without abrogation of Th2 response in the present study is not yet clear to the authors. Detailed understanding of the mechanisms underlying IgE inhibitory property of Den123 is the focus of the future research.

In conclusion, Den123 nanoparticles show a promising IgE-inhibitory property while maintaining Th1/Th2 balance after DNA vaccination. It was indicated that Den123 nanoparticles result in sustained release of DNA plasmids which augmented the IgG2a level gradually until the end of experiment. This does not prove that Den123 nanoparticles have direct effect on IgG2a level but indicates their indirect effect via prolonging the presence of pCMV-Betv1 intracellularly. Also it was clearly shown that 10 µg plasmid with or without Den123 does stimulate the immune response for prevention of allergic disease though not to the same extent as 100 µg dose does. Footpad administration of DNA vaccines three times with 2-week intervals was shown to be a proper site/route/method and schedule for preventive approach in DNA vaccination against allergy.

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