

Immunization with a low-dose replicon DNA vaccine encoding Phl p 5 effectively prevents allergic sensitization

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Background: Replicase-based DNA vaccines stimulate T_H1-biased immune responses at ultralow doses and induce self-removal of transfected cells through apoptosis. Both aspects are important requirements for efficient and safe DNA-based immunotherapy of type I allergies. **Objective:** A Sindbis virus replicon-based DNA vaccine encoding the major timothy grass pollen allergen Phl p 5 was evaluated for its antiallergic potential compared with a conventional DNA vaccine in a BALB/c mouse model of allergy. **Methods:** Mice were intradermally prevaccinated with plasmid DNA, followed by sensitization and intranasal allergen provocation with recombinant Phl p 5. *In vitro* proliferation and cytokine secretion was measured in splenocyte cultures. **Distribution of IgG1, IgG2a, and IgE antibody subclasses** was determined by means of ELISA. **IgE-mediated degranulation** was measured with the basophil release assay. **Bronchoalveolar lavage fluid** was analyzed for eosinophils, IL-4, IL-5, IL-13, and IFN- γ . **Mucus production, inflammatory infiltrates, and epithelial damage** were determined in lung sections. **Results:** Both vaccines induced T_H1-biased immune responses, resulting in suppression of functional IgE, reduction of eosinophilia in bronchoalveolar lavage fluid, and alleviation of lung pathology. However, immunization with the replicon DNA vaccine elicited these effects at a 100-fold lower dose compared with the conventional DNA vaccine.

Conclusions: The increased immunogenicity of replicon-based DNA vaccines allows for application of extremely low doses, thereby eliminating the concerns associated with conventional DNA vaccines, which have to be administered at milligram amounts to induce immune reactions in human subjects. **Clinical implications:** Their high safety profile makes replicon-based DNA vaccines promising candidates for treatment of type I allergies in the clinic. (J Allergy Clin Immunol ■■■■; ■■■:■■■-■■■.)

Key words: Type I allergy, DNA vaccine, genetic immunization, replicase, alphavirus, Phl p 5, IgE, bronchoalveolar lavage, eosinophils, lung pathology

Vaccination with plasmid DNA has proved its efficacy in animal models of viral diseases,¹ as well as bacterial^{2,3} and parasitic^{4,5} infections. Because of their potential to induce strong and long-lasting T_H1-type immune responses, DNA-based vaccines have also been used for the treatment of allergy⁶⁻⁸ and cancer.^{9,10} However, despite some success in clinical applications,¹¹ results from preclinical work with primates, as well as clinical trials with DNA vaccines, suggest that DNA immunization might be less effective in human subjects than expected.^{12,13} The immunogenicity of DNA vaccines depends, probably in addition to other and still unknown factors, on immune activation through recognition of unmethylated CpG dinucleotides within the bacterial plasmids¹⁴ and subsequent signaling through Toll-like receptor (TLR) 9.¹⁵ Differences in the responsiveness of mice and primates to plasmid DNA-related danger signals might account for the poor efficacy in clinical studies.¹⁶ Human monocytes,¹⁷ as well as monocyte-derived dendritic cells,¹⁸ have been reported to lack sensitivity to CpG stimulation. However, recent studies with TLR9 knockout mice even raise the question of whether TLR9 plays any role for the immunogenicity of DNA vaccines.¹⁹

In contrast, replicase-based DNA vaccines deliver qualitatively different immune activating signals by mimicking a viral infection. Among these virus-derived danger signals, double-stranded RNA intermediates generated during alphaviral replication provide a TLR9-independent danger signal.^{20,21}

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Supported by the Austrian Science Foundation, project no. S8811, S8813, and T133-B08; the CeMM project of the Austrian Academy of Sciences; and the Ludwig Boltzmann Institute for Experimental Surgery.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication July 22, 2005; revised April 19, 2006; accepted for publication April 27, 2006.

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0091-6749

doi:10.1016/j.jaci.2006.04.048

Abbreviations used

BAL:	Bronchoalveolar lavage
H&E:	Hematoxylin and eosin
PAS:	Periodic acid–Schiff
pCMV:	DNA expression vector without insert
pCMV-P5:	DNA expression vector encoding Phl p 5
pSin:	Sindbis replicon-based DNA expression vector without insert
pSin-P5:	Sindbis replicon-based DNA expression vector encoding Phl p 5
TLR:	Toll-like receptor

Replicon vectors encode an alphaviral replicase, an antigen of interest substituting for the genes encoding the structural virus proteins and a strong promoter to initiate transcription in the nucleus.²² This type of vaccine proved to be superior compared with conventional plasmid vectors at inducing cellular, as well as humoral, immune responses.^{20,23–25} One of the mechanisms underlying the superior immunogenicity of self-replicating vaccines seems to be activation of professional antigen-presenting cells through enhanced uptake of antigen from transfected cells^{26,27} that die apoptotically^{20,21} because of antiviral defense mechanisms.²⁵ Two features clearly distinguish replicon DNA vaccines from conventional DNA vaccines: the extraordinary immunogenicity and the short-term expression of the plasmid resulting from apoptotic death of transfected cells. Fulfilling 2 requirements for clinical application, specifically low-dose vaccination and high safety, replicon DNA vaccines appear to be ideal candidates for DNA immunization approaches against allergic diseases.

In the current study we analyzed the potential of replicase-based and conventional DNA vaccines to protect mice from induction of anaphylactic IgE, eosinophilia in bronchoalveolar lavage (BAL) fluid, and pathologic changes in the lung tissue after subsequent sensitization with allergen. For the first time, we demonstrate that a replicase-based DNA vaccine encoding the clinically relevant major timothy grass pollen allergen Phl p 5 elicits antiallergic effects similar to a conventional plasmid vector at a 100-fold lower dose.

METHODS

Plasmids

The DNA sequence encoding Phl p 5 was subcloned from λ gt11 cDNA²⁸ into expression vector pCI (Promega, Mannheim, Germany) by using standard molecular methods, and the resulting vector was designated pCMV-P5. The cloning of a Sindbis replicon-based DNA expression vector without insert (pSin) from the commercially available vectors pCI and pSinRep5 (Invitrogen, Lofer, Austria) has been described in detail.²⁹ The coding sequence of Phl p 5 was inserted into the *Xba*I site of pSin to generate pSin-P5.

Mice, immunization, and serology

Female BALB/c mice (6–8 weeks of age; Charles River, Sulzfeld, Germany) were immunized intradermally 3 times at weekly intervals

with various doses (0.01–100 μ g) of pCMV-P5, pSin-P5, or empty mock vectors (0.1–100 μ g pCMV, pSin). Control animals were left untreated until sensitization. Four weeks after the last immunization, mice were sensitized by means of 3 subcutaneous injections of 5 μ g of recombinant Phl p 5 (Biomay, Vienna, Austria) in sterile PBS with 100 μ L of Al(OH)₃ (alum; Serva, Heidelberg, Germany) in a total volume of 200 μ L in 2 spots on the back. The first 2 sensitization rounds were applied at a 2-week interval, and the third sensitization was performed 1 week after the second sensitization. Three weeks after the last sensitization, lung pathology was induced by means of intranasal application of 5 μ g of Phl p 5 in 50 μ L of PBS on 3 consecutive days. Additional groups of mice were killed 2 weeks after the third DNA immunization to assess the prechallenge cytokine secretion status of splenocytes. All animal experiments were conducted according to local guidelines approved by the Austrian Ministry of Science.

IgG1, IgG2a, and IgE antibody levels in sera taken at regular intervals were determined by using a luminescence-based ELISA, as previously described.³⁰

Lymphocyte cultures

Lymphoproliferation of splenocytes was measured as previously described (except that 1% FCS instead of 5% FCS was used and cells were plated at a density of 2×10^5 cells per well).³¹ IL-4, IL-5, IL-10, IL-13, and IFN- γ levels were measured in culture supernatants by using a sandwich ELISA (BD Biosciences, Schwench, Austria and R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions.

β -Hexosaminidase release from rat basophil leukemia cells

As a functional read out for IgE-mediated degranulation, a β -hexosaminidase release assay was performed by using the rat basophil leukemia cell line RBL-2H3, as previously described.³¹

Analysis of BAL fluid

BAL was carried out with 2×1 mL of fresh, ice-cold PBS by using a hypodermic needle. Samples were centrifuged through a 40- μ m cell strainer (BD Biosciences), and supernatants were stored for further analysis of cytokines. Erythrocytes were lysed for 10 minutes at room temperature with 500 μ L of FACS-Lyse (BD Biosciences). Cells were washed in FACS-Buffer (PBS, 0.5% BSA, and 2 mM EDTA), resuspended in 1 mL of FACS-Buffer, labeled with anti-CD45 FITC/anti-Gr1 PE (BD Biosciences) on ice for at least 10 minutes, and analyzed on a FACSCalibur flow cytometer. Eosinophils were distinguished from other leukocyte populations by their CD45^{med} Gr1^{low} side-scatter^{high} phenotype, and their relative amount compared with total CD45⁺ cells was calculated. Results from flow cytometry were confirmed by means of standard histologic analysis of cytospin preparations.

Lung histology

Lungs were fixed in 4% formalin at 4°C overnight and embedded in paraffin. Tissue sections of 2 μ m were stained with hematoxylin and eosin (H&E) dye or periodic acid–Schiff (PAS) by using standard protocols. Immunostaining of Ki-67 was performed with TEC-3 antibody (Dako, Vienna, Austria) by using the streptavidin-biotin-peroxidase complex method. Briefly, tissue sections were deparaffinized and watered; endogenous peroxidases were blocked with 3% H₂O₂-methanol. Tissue sections were then autoclaved in citrate buffer and incubated with rabbit serum. Primary antibody was diluted 1:25 in standard diluent (Dako) and incubated at 4°C overnight. Secondary biotinylated antibody was diluted in PBS (1:200) and incubated for 30 minutes. Slides were developed with diaminobenzidine and counterstained with H&E.

All sections were analyzed by means of light microscopy by an investigator who was blind to the treatments. Severity of infiltrates in H&E sections was scored from 1 (no or mild infiltrate) to 4 (severe infiltrate). In PAS sections the number of goblet cells in bronchioles was determined to detect goblet cell metaplasia. Proliferating epithelial cells in bronchioles were counted in TEC-3-stained sections.

Statistical analysis

Statistical differences between means of P5 DNA-immunized groups and the respective mock vector-immunized groups were assessed by using the Student *t* test. The 0.01 μ g P5 DNA-immunized groups were compared with nonpreimmunized control animals. Groups that failed normality tests and scored H&E sections were analyzed by using the Mann-Whitney rank sum test.

RESULTS

Intradermal injection of replicon-based DNA vaccine encoding Phl p 5 induces a T_H1 -biased immune response at a 100-fold lower dose compared with a conventional DNA vaccine

To characterize the immune response type induced by a Sindbis replicase-based vaccine encoding Phl p 5 (pSin-P5) compared with a conventional DNA vaccine, pCMV-P5, mice ($n = 5$) immunized with increasing doses (0.01–1 μ g) of pSin-P5 or pCMV-P5 were killed; splenocytes were prepared and restimulated with recombinant Phl p 5. Measurement of tritiated thymidine uptake (Fig 1, A) indicated that pCMV-P5 induced significant proliferation compared with splenocytes from naive animals only at the highest dose applied (1 μ g), whereas the replicon-based vaccine pSin-P5 induced significant proliferation at all tested doses (*P* values ranging from .004 to <.001). Cytokines were measured in proliferation supernatants to determine the T_H1/T_H2 profile. Again, pSin-P5 induced significant amounts of IFN- γ at all tested doses (*P* values ranging from .022 to <.001), whereas only the highest dose of pCMV-P5 resulted in significant IFN- γ levels (*P* < .01). No IL-4, IL-5, IL-10, or IL-13 could be detected (data not shown). Taken together, these data demonstrate that the replicon-based vaccine can induce a clearly T_H1 -biased antigen-specific immune response at a 100-fold lower dose compared with the conventional DNA vaccine.

Low-dose immunization with the replicon DNA vaccine pSin-P5 prevents induction of IgE antibodies after sensitization

Mice ($n = 6$) prevaccinated with plasmid DNA were sensitized by means of 3 subcutaneous injections with recombinant Phl p 5 in alum, a protocol yielding high levels of IgE antibodies.³⁰ Phl p 5-specific IgG1 and IgG2a levels were measured after DNA vaccination (Fig 2, A; serum dilution, 1:5000) and after sensitization (Fig 2, B; serum dilution, 1:20,000). Although pCMV-P5 induced significant levels of Phl p 5-specific IgG1 and IgG2a (*P* < .005) only at doses of 1 μ g or higher,

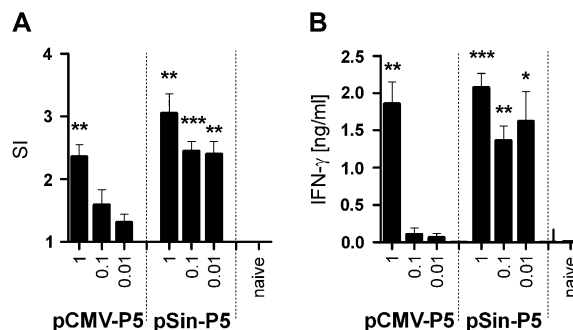


FIG 1. Cellular immune responses against Phl p 5. **A**, Stimulation indices (SI) calculated from tritiated thymidine uptake. **B**, IFN- γ in supernatants of restimulated splenocytes. Data are displayed as means $\pm$ SEM. **P* < .05, ***P* < .01, ****P* < .001.

pSin-P5 induced significant (*P* < .005) IgG1 and IgG2a antibody production at all tested doses compared with that seen in mock-immunized or naive (not shown in graph) control animals (Fig 2, A). After sensitization, nonimmunized, as well as mock-immunized, control animals displayed high levels of IgG1 and virtually no IgG2a. In contrast, preimmunization with either DNA construct induced significant suppression of IgG1 (*P* values ranging from .021 to <.001) compared with that seen in the respective mock-immunized groups, except for the pCMV-P5 100 group, in which suppression was barely not significant when compared with the mock effect (*P* = .06). All mock vector groups showed a slight but significant suppressive effect on IgG1 compared with that seen in nonprevaccinated control animals (*P* values ranging from .042 to <.001), except for the pSin 10 group (*P* = .32). Similarly, whereas all groups prevaccinated with pSin-P5 showed increased IgG2a titers, pCMV-P5-immunized mice displayed significantly higher IgG2a levels than the mock-immunized groups only at doses of 1 μ g or higher (*P* values ranging from .002 to <.001). Mock vectors induced no statistically significant IgG2a compared with nonprevaccinated control animals. These data indicate that previously induced T_H1 cells are further expanded by subsequent sensitization and can deviate the immune response toward a T_H1 -type reaction. This was also confirmed by means of analysis of cytokines in supernatants from restimulated splenocyte cultures (see Fig E1 in the Online Repository at www.jacionline.org). This immune deviation resulted in a significant suppression of IgE on sensitization compared with that seen in the corresponding mock-immunized animals (*P* values ranging from .002 to .001; Fig 3, black bars), except in the groups that had received low doses (0.01 and 0.1 μ g) of the conventional DNA vaccine, as detected with an RBL assay (serum dilution, 1:100), an *in vitro* correlate for antigen-specific cross-linking of IgE. In contrast to both DNA vaccines, which conferred full protection from functional IgE expression, no significant effect of the empty vectors could be detected, indicating a negligible influence of unspecific

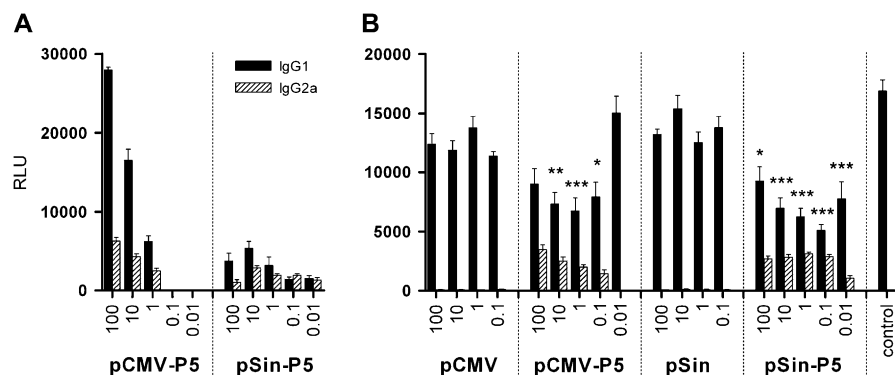


FIG 2. Phl p 5-specific antibody responses. IgG1 and IgG2a antibody levels of mice immunized with the different DNA vaccines at various doses before (A) and after (B) sensitization with recombinant allergen. Data are presented as means $\pm$ SEM. *P* values for IgG1 suppression are as follows: **P* < .05, ***P* < .01, ****P* < .001. RLU, Relative light units.

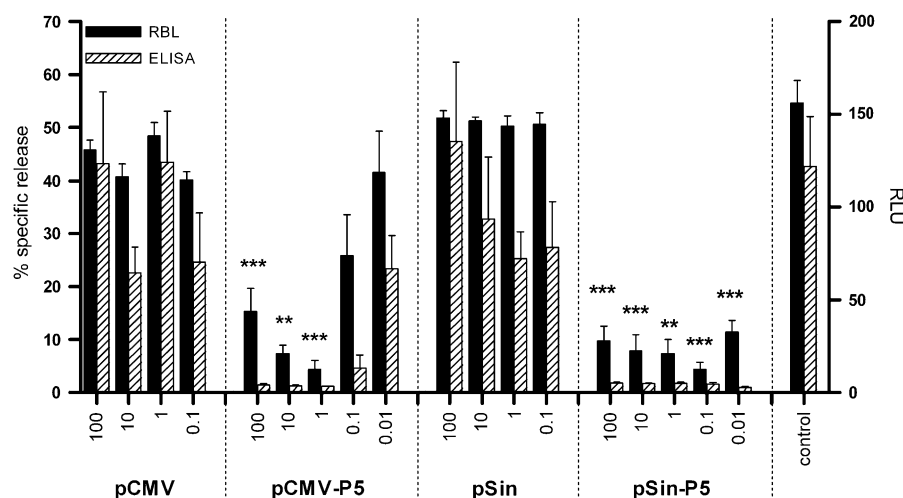


FIG 3. Phl p 5-specific IgE responses after sensitization. Biologically active IgE was measured by using the RBL release assay (filled bars). Data were confirmed by means of ELISA (hatched bars). Data are presented as means $\pm$ SEM. *P* values for RBL results are as follows: **P* < .05, ***P* < .01, ****P* < .001. RLU, Relative light units.

CpG-mediated effects. Similar data were obtained by using a Phl p 5-specific IgE ELISA (serum dilution, 1:100; Fig 3, hatched bars).

Prevaccination with the replicon DNA vaccine alleviates pathologic alterations in the lung induced by intranasal challenge

Sensitized mice were challenged through 3 daily intranasal applications of 5 μ g of recombinant Phl p 5 to estimate the effect of DNA vaccination on lung inflammation. This treatment results in invasion of eosinophils into the lung tissue, which is reflected by a high percentage of eosinophils in BAL fluid (Fig 4, A), as well as increased levels of IL-5 and IL-13 (Fig 4, C and D). Prevaccination with either DNA vaccine significantly reduced the amount of eosinophils by 50% to 60% compared with that seen in mock-immunized or control animals (*P* < .001; pSin-P5

100, *P* = .014), except for the low doses of pCMV-P5 (0.1-0.01 μ g) that induced lower (16% to 21%) but still significant (*P* = .041) reduction of eosinophils. Similarly, levels of IL-5 in BAL fluid were suppressed in a clearly dose-dependent manner by means of prevaccination with pCMV-P5, with no significant effect in the 0.01- μ g group (*P* values ranging from .002 to <.001). In contrast, pSin-P5 significantly suppressed IL-5 levels over the whole range of tested doses (*P* values ranging from .015 to <.001). Although a significant induction of IL-13 (Fig 4, D) could be measured in sensitized animals (*P* = .005), no differences between groups were observed, regardless of the pretreatment. DNA vaccination with either construct induced low levels of IFN- γ in BAL fluid in some animals, but this effect was inconsistent through the groups (Fig 4, B).

Additionally, lungs were analyzed for goblet cell metaplasia by means of microscopic cell counts on

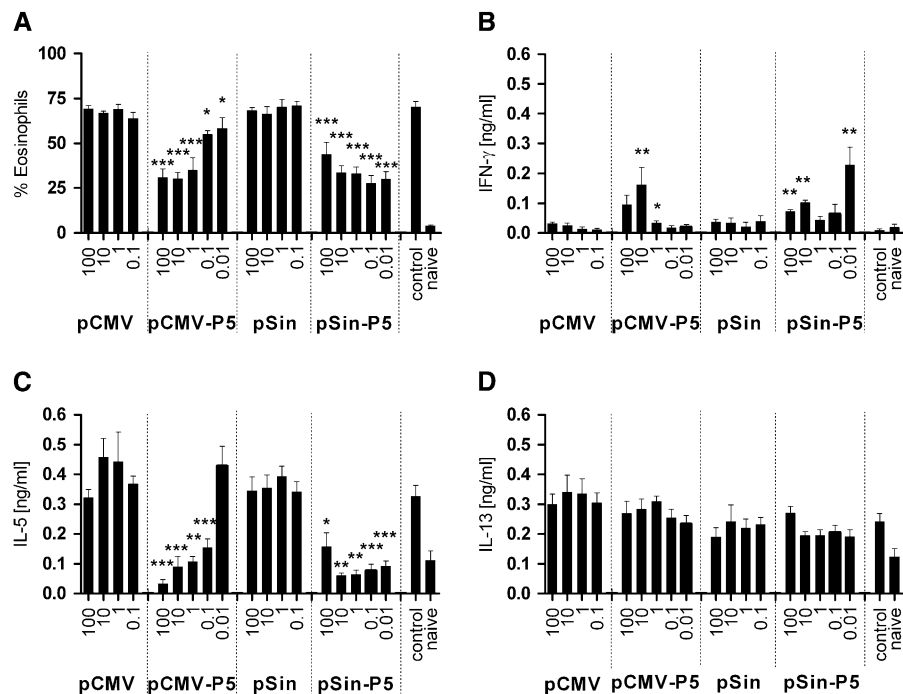


FIG 4. Analysis of BAL fluid. Percentage of eosinophils in BAL fluid (A) and IFN- γ (B), IL-5 (C), and IL-13 (D) levels in BAL fluid. Data are shown as means $\pm$ SEM. *P* values compared with respective mock groups are as follows: **P* < .05, ***P* < .01, ****P* < .001.

PAS-stained paraffin sections (Fig 5, A) and Ki-67 immunostaining (Fig 5, B) as markers for epithelial damage in the lung. For both parameters, a similar reduction compared with eosinophil reduction in BAL fluid could be observed. PAS staining showed a significant reduction of mucus-secreting goblet cells in all DNA-vaccinated groups, except for the pCMV-P5 0.01 group (*P* values ranging from .023 to .002). Reduction of Ki-67⁺ epithelial cells in the bronchus was significant for all doses of the replicon-based DNA vaccine (*P* values ranging from .037 to .002) but only for the groups receiving 10 and 100 μ g of pCMV-P5 (*P* < .05) compared with the corresponding mock vector groups. No significant differences could be observed concerning the severity of infiltrate on H&E-stained sections; however, the trend was similar to PAS and Ki-67 results in the respective prevaccinated groups (see Table E1 in the Online Repository at www.jacionline.org). Nevertheless, similar to the observed differences in BAL fluid, the ratio of eosinophils to lymphocytes was reduced by prevaccination (data not shown). Representative sections for each infiltration score of either prevaccinated or nonvaccinated control animals are shown in Fig E2 (see the Online Repository at www.jacionline.org). Additionally, we measured increases in enhanced pause as an index of airway obstruction by using whole-body plethysmography (PLY3211-unrestrained whole-body plethysmograph; Buxco, Winchester, United Kingdom) in selected animals and could show that at low doses, pSin-P5 is superior to pCMV-P5 in reducing enhanced pause compared with results in mock-immunized

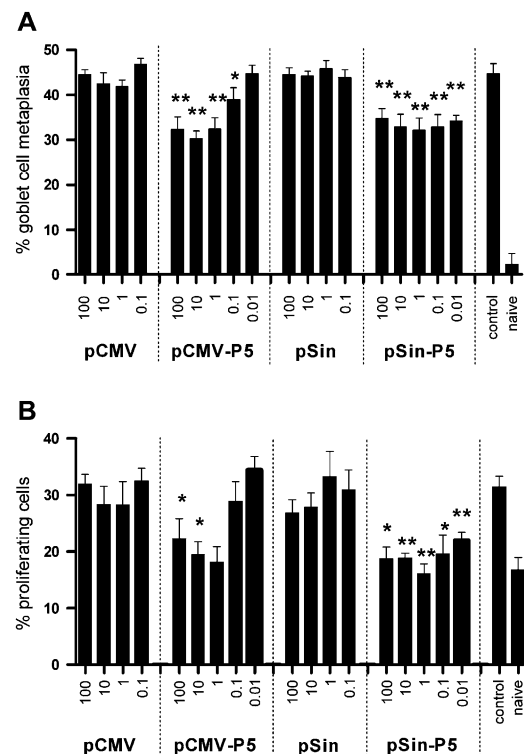


FIG 5. Lung histology from challenged animals. A, Percentage of mucus-producing goblet cells. B, Percentage of proliferating bronchial epithelial cells. Data are shown as means $\pm$ SEM. *P* values compared with respective mock groups are as follows: **P* < .05, ***P* < .01, ****P* < .001.

mice (see Fig E3 in the Online Repository at www.jacionline.org).

DISCUSSION

Currently, the only preventive or even curative approach for treatment of type I allergy is allergen-specific immunotherapy, which has several disadvantages, such as low efficacy for patients with allergic rhinitis or asthma, the use of scarcely standardized crude allergen extracts, the risk of cross-sensitization, and anaphylactic side effects during treatment (reviewed in Valenta et al³²).

The effect of DNA immunization for the treatment of type I allergy has been demonstrated in numerous pre-clinical studies. The antiallergic effect of DNA vaccines could be clearly attributed to the recruitment of T_H1 cells and the establishment of a balancing T_H1-biased cytokine milieu,^{6,7,31,33-39} with the exception of one study investigating *in vitro*-created T_H1 and T_H2 cells.⁴⁰ In our current work we investigated for the first time a replicon-based DNA vaccine encoding a clinically relevant allergen, the major timothy grass pollen allergen Phl p 5, and compared this vaccine with a conventional DNA vaccine encoding the same allergen. The immunogenicity of the conventional DNA vaccine was clearly dose dependent and, although Phl p 5 proved to be an exceptionally strong antigen, rapidly decreased at doses of less than 1 µg. With a different antigen (β-galactosidase), immunogenicity was even lost at doses of less than 10 µg (data not shown). In contrast, the immunologic effects of the replicase-based DNA vaccine seemed to be rather dose independent over the entire range of doses tested. It is known from *in vitro* transfection studies that for conventional CMV-based plasmids, the overall expression level directly correlates with the amount of DNA used, leading to the disappearance of highly transfected cells at low DNA concentrations. In contrast, replicon DNA-transfected cells express the same amount of antigen independent of the amount of plasmid used, establishing a quantitative, as well as a qualitative, difference between the 2 systems. This phenomenon could explain why immune responses induced by CMV-based plasmids correlate with the dose of injected DNA, whereas plasmid DNA replicons do not show a dose titration.²⁰

Our experiments showed a T_H1-biased response for both vaccines, as indicated by Phl p 5-specific IFN-γ secretion (Fig 1, B) and the lack of IL-4, IL-5, and IL-13. Furthermore, we found no evidence for recruitment of IL-10-secreting T regulatory cells with both conventional and replicon DNA vaccines. Together with previous results,³¹ this clearly demonstrates that T regulatory cells do not play a significant role in the antiallergic mechanisms of DNA vaccination. This also seems to be valid for replicon-based DNA vaccine types.

However, both DNA vaccines differ in their ability to induce antibodies. Although the replicase-based vaccine only induces low amounts of IgG1 and IgG2a, the conventional DNA vaccine elicits high levels of both

antibody subclasses in a dose-dependent manner. The reduction or even lack of antibody production can be attributed to the specific immune activation mechanisms underlying replicase-based DNA vaccines. Replicon vaccines gain immunogenicity through double-stranded RNA intermediates recognized by TLR3^{41,42} and through induction of immune apoptosis.⁴³ The latter leads to reduced availability of soluble antigen accessible to B cells, thereby accounting for the observed lower antibody titers. In contrast, death by apoptosis increases the uptake of antigen by professional antigen-presenting cells for processing and presentation to T cells.²⁶ Additionally, apoptosis-related reduction or lack of native allergen renders replicon DNA vaccines hypoallergenic because only minute amounts of native allergens are released during the course of vaccination.

Although replicon vaccines show highly reduced induction of antibody production, they still maintain full antiallergic capacity. This confirms previous data showing that DNA vaccines can display antiallergic effects without induction of allergen-specific antibodies solely based on the induction of T_H1-biased antigen-specific T-cell responses.^{31,44} It has been shown that early but transient expression of T_H1 responses is an important marker for successful specific immunotherapy with allergens.⁴⁵ However, in the past years, some studies have suggested that allergen-specific T_H1 responses might lead to acute or chronic T_H1-mediated inflammatory diseases at the sites of reoccurring antigen exposure.⁴⁶⁻⁴⁸ These conclusions were based on experiments that transfer large amounts of monoclonal T-cell receptor-transgenic T_H1 cells and challenge with allergen within a short time interval after application of the T cells. There is no evidence that allergen-specific T_H1 cells, which are directly generated in the vaccinated animals, would also lead to T_H1-associated immunopathology after frequent exposure to allergen. Indeed, we could show that preimmunization with both DNA vaccine types not only prevented IgE induction (Fig 3) but also reduced inflammation in the lung after a subsequent sensitization and intranasal challenge with allergen (Fig 4, A and C, and Fig 5). Although the percentage of eosinophils was reduced, the total amount of infiltrate was not significantly altered (Table E1 and Fig E2 in the Online Repository at www.jacionline.org), indicating that prevaccination modified the quality rather than the quantity of the infiltrate. Pre-treatment with the DNA vaccines not only reduced eosinophilia but also mitigated mucus production and bronchial epithelial damage, as well as reduced airway obstruction (see Fig E3 in the Online Repository at www.jacionline.org). We also found reduced levels of IL-5 and little or no IFN-γ in the BAL fluid of prevaccinated animals. Taken together, these parameters indicate no DNA vaccine-induced immunopathology but rather alleviation of lung pathology.

Summing up, the current study demonstrates that a replicase-based DNA vaccine, applied at a 100-fold lower dose, compared with a conventional DNA vaccine has similar protective efficacy in a prophylactic mouse model

of allergy. The markedly reduced amount of plasmid necessary for induction of antiallergic immune responses will certainly increase the acceptance of DNA vaccination against type I allergies for clinical applications. Moreover, self-removal of replicon-based vaccines through induction of apoptosis of transfected cells adds a measure of safety to naked plasmid vaccines. Because DNA replicons gain their immunogenicity through viral danger signals and induction of antiviral immune response types, they are also potent candidates to overcome the poor immunogenicity of DNA vaccines observed in some clinical studies. Hence this latest generation of DNA vaccines fulfills all the necessary prerequisites for clinical evaluation in allergic patients.

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