Immunize and disappear—Safety-optimized mRNA vaccination with a panel of 29 allergens

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Background: The spread of type I allergic diseases has reached epidemic dimensions. The success of therapeutic intervention is limited, and hence prophylactic vaccination is now seriously considered. However, immunization of healthy individuals requires safety standards far beyond those applicable for therapeutic approaches. mRNAs encoding allergen molecules represent an attractive tool for preventive vaccination because of the inherent safety features of this vaccine type.
Objective: In the current study we investigated whether mRNA constructs would be capable of protecting against type I allergic reactions in a murine model using the grass pollen allergen Phl p 5 and 28 other major pollen, food, animal, mold, and latex allergens.
Methods: BALB/c mice were immunized intradermally either with conventional or replicase-based mRNA constructs. Subsequently, animals were sensitized by means of subcutaneous injection of allergen/alum, followed by airway provocation. IgG1/IgG2a/IgE titers were determined by using ELISAs. Allergen-specific functional IgE levels were assessed by using the basophil release assay. Measurement of cytokines in splenocyte cultures and bronchoalveolar lavage fluids were performed by using enzyme-linked immunosorbent spot assays/sandwich ELISAs. Eosinophil and $CD8^+$ counts in bronchoalveolar lavage specimens were determined by means of flow cytometry. Airway hyperreactivity was assessed with whole-body plethysmography and invasive resistance/dynamic compliance measurement.
Results: mRNA vaccination proved its antiallergic efficacy in terms of IgG subclass distribution, functional IgE suppression, reduction of IL-4 and IL-5 levels, induction of IFN-γ–producing cells, and reduction of airway hyperreactivity and eosinophil counts in the lung.
Conclusion: Immunization with mRNA induces T_H1-biased immune responses similar to those elicited through DNA-based vaccination but additionally offers the advantage of a superior safety profile. (J Allergy Clin Immunol 2009;124:1070-7.)

Key words: *RNA vaccines, DNA vaccines, self-replicating vaccines, type I allergy, vaccination*

During the last decades, type I allergic diseases have emerged as a major public health problem in Western industrialized countries, with about 25% of the population being affected.[1,2] In addition to family predisposition, conditions of growing up and environmental factors have been demonstrated to be of great relevance for the development of atopic diseases.[3-5]

Specific immunotherapy currently represents the only available therapeutic intervention. However, because of the high doses administered, the risk of anaphylactic side effects is evident, and the use of crude, barely characterized allergen extracts implies the possibility for sensitization of the patient against previously unrecognized components.[6,7]

Additionally, no preventive vaccination against type I allergy is available, although immunization of young children with increased hereditary risk of allergic diseases might be the most feasible approach because training of the naive immune system is easier to accomplish than diverting an already manifested allergic immune phenotype. Complete conversion of an established T_H2-type response, especially with respect to IgE production, seems to fail because of a persistent production of specific IgE secreted by plasma cells that survive in appropriate niches. These long-lived plasma cells represent an independent cellular component of immunologic memory.[8]

Recently, nucleic acid–based vaccines have become a promising approach to bias immune mechanisms underlying allergic diseases. It has been shown in numerous animal studies that DNA vaccines can prevent the induction of type I allergic responses and even downregulate an already established allergic T_H2 immune status.[9] Nevertheless, concerns have been raised regarding the safety of DNA-based vaccines with respect to integration of the plasmid DNA into the host genome, adverse immunopathologic effects, induction of autoimmune diseases, and uncontrollable long-term expression of the encoded antigen.[10,11]

Especially with respect to a vaccine against a not imminently life-threatening disease and considering prophylactic vaccination

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Abbreviations used
ELISPOT: Enzyme-linked immunosorbent spot
Penh: Enhanced pause
P5-RNA: mRNA encoding Phl p 5
P5-repRNA: Self-replicating mRNA encoding Phl p 5
TLR: Toll-like receptor

of healthy children, the safety of any antiallergic vaccine is of utmost importance. We therefore specify the requirements for an antiallergic vaccine as a minimal construct that is eliminated from the body after the induction of a desired immune response. We call this concept "immunize and disappear."

A type of nucleic acid that could probably meet these enhanced safety requirements is mRNA. mRNA vaccines exhibit all the advantages attributed to DNA vaccines for the treatment of allergic diseases. They provide the allergen in its purest form—its genetic information—and at such low doses that neither induction of anaphylactic side effects caused by pre-existing IgE nor production of any novel allergen-specific IgE will occur.

In addition, mRNA vaccines offer several advantages over DNA vaccines. First, only the genetic information of the allergen itself has to be used, whereas no additional foreign sequences, such as plasmid backbone DNA or viral promoters, are included, thereby fulfilling the requirements of a minimal construct. Second, mRNA cannot integrate into the host genome, abolishing the risk of malignancies. Third, mRNA is translated in the cytoplasm of the cell, and hence the transcription machinery of the cell nucleus is not needed, rendering mRNA vaccines independent of transport into and out of the nucleus as well as of nuclear stages. Finally, because of the fast degradation of mRNA, expression of the foreign transgene is short-lived.[12]

However, because of the instability of mRNA, it was assumed that high amounts of the vaccine might be needed. Self-replicating mRNA vaccines have been developed to circumvent a possible dosage problem. Such vectors include replication elements derived from alphaviruses, whereas the structural virus proteins have been substituted for the gene of interest. Replicase-based mRNA vaccines induce antibody responses as well as cytotoxic responses at extremely low doses because of immune activation mediated by virus-derived danger signals.[13]

Although a number of articles deal with naked mRNA or mRNA replicon vaccines in the field of tumor immunology, no study investigating the potential of mRNA vaccines for prevention or treatment of type I allergy has been published thus far. In the current study, using the timothy grass pollen allergen Phl p 5, we exemplify for the first time that mRNA and replicase-based mRNA vaccines can effectively prevent allergic responses and represent promising tools for specific allergy immunization. In addition, we provide proof of principle for the wide applicability of this method by using a panel of 28 food, pollen, latex, dander, and mold allergen–encoding mRNAs.

METHODS

Plasmids used for mRNA transcription

The Phl p 5 gene was excised from vector pCMV-Phlp5[14] by *Nhe*I/*Xba*I and ligated into the *Xba*I restriction site of pTNT (Promega, Madison, Wis) and pSin-Rep5 (Invitrogen, Carlsbad, Calif), resulting in pTNT-P5 and pSin-Rep5-P5, respectively. Similarly, a panel of 28 food, pollen, latex, dander, and mold allergens has been cloned into pTNT (see Table E1 in this article's Online Repository at www.jacionline.org). All inserts were sequence verified (Eurofins MWG Operon, Ebersberg, Germany). Sequences of all allergens used for RNA vaccination are shown in Table E2.

RNA transcription

Plasmids pTNT-P5 and pSin-Rep5-P5 were linearized, and templates were purified by means of phenol-chloroform-isoamyl alcohol extraction, followed by a single chloroform-isoamyl alcohol extraction. After addition of a 1:10 volume of 3 mol/L Na-acetate, pH 5.2, plasmids were precipitated with 2 volumes of 100% ethanol and washed 3 times with 70% ethanol.

All transcription reactions were performed with T7 or SP6 RiboMAX Large Scale RNA Production Systems (Promega). Residual template DNA was removed by means of digestion with RNAse-free DNAse (Promega).

RNAs were capped *in vitro* by using a ScriptCap m7 G Capping System (Epicentre Biotechnologies, Madison, Wis), according to the manufacturer's protocol, to maintain the stability and translational efficiency of the mRNA *in vivo*, except that we used 0.8 μL of capping enzyme (instead of 4 μL) and incubated at 37°C for 2 hours. mRNA was recovered by means of ammonium acetate precipitation, washed with 70% ethanol, and resuspended in nuclease-free H_2O.

Mice, immunization, and serology

Female BALB/c mice (Charles River, Wilmington, Mass) were immunized with different doses of mRNA or self-replicating mRNA in 200 μL of PBS (Phl p 5 groups) or Ringer solution (102.7 mmol/L sodium chloride, 5.4 mmol/L potassium chloride, and 1.8 mmol/L calcium chloride; supplementary groups) 3 times in weekly intervals. The vaccine was applied by means of intradermal injection into the shaved back divided between 6 to 8 different spots. Two weeks later, mice were sensitized by means of subcutaneous injection of 1 μg of recombinant allergen together with 100 μL of $Al(OH)_3$ (Serva Electrophoresis, Heidelberg, Germany) in a total volume of 200 μL of PBS 2 times in 2-week intervals. Sera were taken before and 2 weeks after sensitization. Two weeks later, mice underwent an airway challenge by means of intranasal instillation of 1 μg of recombinant allergen in 40 μL of PBS 3 times in daily intervals (see Fig 1 for the experimental schedule). A shortened schedule was used for groups receiving any of the other 28 allergens tested (see Table E1). All animal experiments were conducted according to local guidelines approved by the Austrian Ministry of Science.

Data are shown for 1 experiment with 40, 8, and 1.6 μg for conventional RNA and 8, 1.6, and 0.32 μg for self-replicating RNA. Data of Figs 2 to 4 have been reproduced in 2 additional independent experiments by using either single doses (40 μg for mRNA encoding Phl p 5 [P5-RNA] and 8 μg for self-replicating mRNA encoding Phl p 5 [P5-repRNA]) or a dose range (100, 10, and 1 μg) that produced comparable results for the respective doses.

Antigen-specific IgG1, IgG2a, and IgE antibody levels in sera were determined by using a luminescence-based ELISA, as previously described.[15] Total IgE levels were measured with an IgE ELISA max kit (BioLegend, San Diego, Calif). Functional IgE levels were measured by using a basophil degranulation assay, as previously described.[16]

Lymphocyte cultures

Splenocytes from both immunized and control mice were cultured to harvest supernatants for IFN-γ and IL-5 sandwich ELISAs (BD PharMingen, San Diego, Calif) and for IL-13 sandwich ELISA (R&D Systems, Minneapolis, Minn) or for IL-4, IL-5, and IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay, as previously described.[14,17] Splenocytes were cultured in the presence or absence of 10 μg/mL recombinant allergen (Biomay AG, Wien, Austria). All allergen preparations contained less than 5 pg of LPS per microgram, as determined by using the Limulus amoebocyte assay.

Analysis of bronchoalveolar lavage specimens

Bronchoalveolar lavage was performed as previously described.[14] Cells were stained with CD45-PECy7, Gr1-allophycocyanin, CD8-phycoerythrin, and CD4-PECy5 (BD PharMingen) and analyzed on a FACSCanto II flow cytometer. Eosinophils were distinguished from other leukocyte populations by their $CD45^{med}$ $Gr1^{low}$ side-$scatter^{high}$ phenotype.