

Inhibition of IgE formation and allergic inflammation by allergen gene immunization and by CpG motif immunostimulatory oligodeoxynucleotides

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Allergen immunotherapy, the subcutaneous injection of small quantities of allergen extract, has been used for many years for the treatment of allergic diseases. Currently, immunotherapy is no longer a major form of allergy therapy because the risk-to-benefit ratio is not tolerable as compared to that of present-day pharmacologic treatment (1). However, if a form of immunotherapy could be found with a much lower risk-to-benefit ratio, immunotherapy would probably regain an important role in the treatment of allergic disorders. Unlike merely treating symptoms with antiallergic drugs, immunotherapy can change the course of the disease by downregulating the allergic immune response, resulting in a long-lasting alleviation of allergic manifestations and often in the cure of allergic diseases. Recently, it was discovered that animals and human subjects can be vaccinated with plasmid DNA (pDNA) encoding an antigen (gene vaccination) (2–4). This form of immunization could also be used for allergen vaccination. It would have a great advantage over conventional immunotherapy because the allergens are produced in the host's cells and are mainly intracellular; therefore, they would not cause anaphylactic reactions, the major risk of allergen-injection immunotherapy. Because gene vaccination for

infectious agents resulted in Th1 immune responses, we determined in mice whether immunization with pDNA encoding protein antigens or allergens also induces Th1 responses. This would be contrary to the Th2 and IgE antibody response that is usually elicited by protein antigens in mice.

Gene vaccination

Intramuscular (i.m.) or intradermal (i.d.) injection of pDNA encoding a specific antigen (gene vaccination) induces both an antibody and a cytotoxic T-lymphocyte (CTL) response (2–4). This was shown in many species, including nonhuman primates, and clinical trials in man for infectious diseases such as the acquired immunodeficiency syndrome (AIDS) are presently being conducted. Initially, the pDNA constructs were injected i.m., and the production of antigen induced by the pDNA was shown in the transfected muscle cells. However, pDNA was also taken up by other cells in the muscle such as antigen-presenting cells, and these cells may have played a more important role than the muscle cells in the induction of the immune response to the encoded antigen (3).

Therefore, we compared the immune response to i.m. or s.c. injected pDNA encoding several

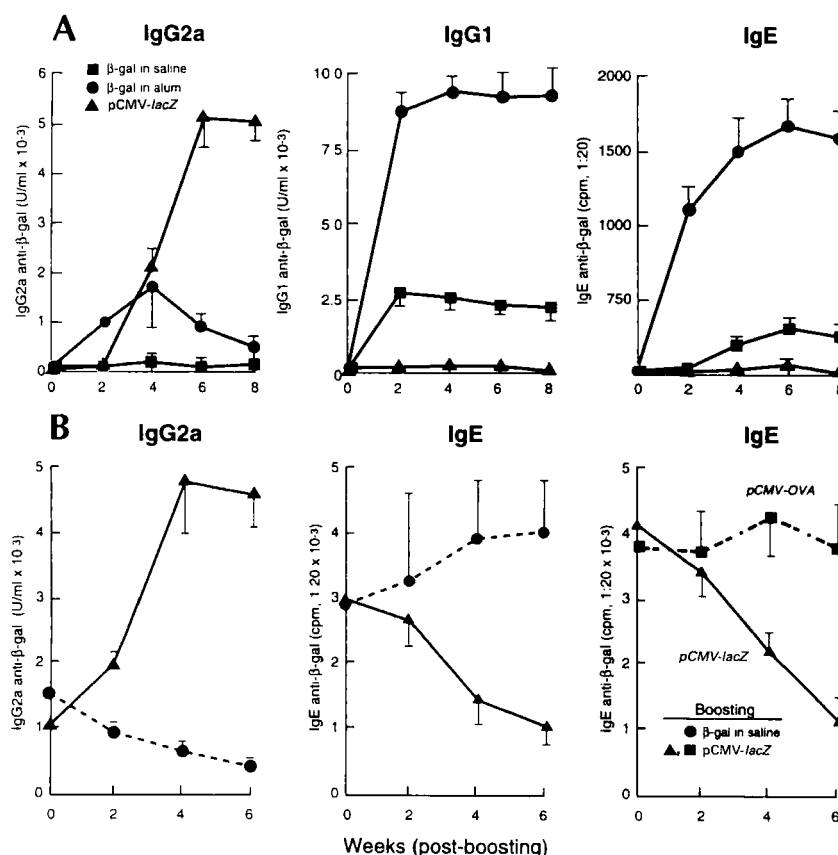


Fig. 1. A) Primary IgG2a, IgG1, and IgE response of BALB/c mice injected with 100 μ g of pCMV-lacZ ($\blacktriangle$), 10 μ g β -gal in saline ($\blacksquare$), or 1 μ g of β -gal in alum ($\bullet$). B) IgG2a, IgG1, and IgE response of β -gal (1 μ g) in alum-primed mice to secondary injection of pCMV-lacZ (100 μ g) ($\blacktriangle$). Control mice were boosted with β -gal (10 μ g) in saline ($\bullet$). For antigen specificity control, mice were boosted with 100 μ g of pCMV-OVA ($\blacksquare$). Results are mean $\pm$ SEM of four mice per group.

antigens and found that s.c. injection is a more effective way of gene immunization than the i.m. route. Intradermal gene immunization is probably more effective than i.m. immunization because the skin contains many more professional antigen-presenting cells than the muscle, especially many Langerhans' cells that are closely related to the very efficient antigen-presenting dendritic cells. It was indeed shown that bone-marrow-derived antigen-presenting cells, rather than somatic cells such as myocytes and keratinocytes, dictate the nature of the immune response to pDNA-encoded antigens (5). Of the many different pDNA constructs tested, pDNA containing a cytomegalovirus (CMV)-based vector with the bovine growth hormone poly-A termination site yielded the best antigen expression (3). By using the *lacZ* gene encoding *E. coli* β -galactosidase (β -gal), we showed by its enzymatic activity that β -gal was produced by transfected cells for at least 17 months after three i.d. pDNA injections. If such a long-lasting response could also be achieved with allergens in man, it would make allergen gene vaccination very attractive as a new form of immunotherapy, especially if it would cause a Th1 response to allergens.

Gene vaccination induces a Th1 response to protein antigens and allergens

To determine whether allergen gene vaccination induces a Th1 or a Th2 response to protein antigens or allergens in mice, we first compared the primary immune response to β -gal with the response to a pDNA construct containing *lacZ*, the gene encoding β -gal (6, 7). The antibody isotype response to β -gal was assessed serially for 8 weeks, and the cytokine release by *in vitro* antigen-activated CD4⁺ splenic T cells was measured 9 weeks after priming. As shown in Fig. 1A, immunization with β -gal in saline or alum induced high titers of IgG1 and IgE antibodies, both isotypes being typical of murine Th2 responses, whereas these mice produced only low levels of IgG2a antibodies, the isotype which is typical of a Th1 antibody response (8). In contrast to protein immunization, pCMV-lacZ-vaccinated mice displayed the reciprocal antibody isotype pattern with high IgG2a (15-fold higher than protein in alum-injected mice at 8 weeks), low IgG1 levels (30-fold lower than β -gal in alum-immunized mice), and no IgE antibodies. To ensure that the lack of detection of IgE anti-

bodies in pCMV-*lacZ*-immunized mice was not the result of competition for antigen in the IgE RAST assay by the high levels of IgG2a anti- β -gal antibodies, the IgG was removed by protein G-Sepharose absorption, and the sera were retested for IgE antibodies. Removal of IgG increased the IgE titer in the β -gal-immunized mice, but removal of the IgG still did not reveal significant levels of IgE antibodies in the gene-vaccinated animals. Mice primed with pDNA also had very low total IgE serum levels (130 ng/ml), 13% of the levels of the β -gal in alum-injected mice (1030 ng/ml) and 22% of the levels of the β -gal in saline-primed mice (590 ng/ml). These data suggested that pDNA immunization induced a Th1 response and protein immunization a Th2 response to β -gal. This was confirmed by measuring the lymphokine secretion of *in vitro* activated CD4⁺ spleen cells 9 weeks after immunization. The T cells of the pCMV-*lacZ*-injected mice secreted interferon- γ (IFN- γ) and no interleukin-4 (IL-4) and IL-5, whereas the cells from the protein-primed mice secreted IL-4 and IL-5 but no IFN- γ (6).

Primary gene vaccination inhibits the subsequent induction of a Th2 response

In the next set of experiments, we determined whether boosting with β -gal in alum can switch the Th1 anti- β -gal response in pCMV-*lacZ*-primed mice toward a Th2 response. For this purpose, pCMV-*lacZ*-primed mice were boosted with β -gal in alum 4 weeks after the primary immunization. Protein boosting increased the pre-existing IgG2a anti- β -gal titer 10-fold in the pDNA-primed mice. However, β -gal in alum did not induce significant amounts of IgE antibodies, in contrast to the β -gal in saline- or alum-primed mice, which showed the expected secondary IgE response. This shows that the pDNA-primed mice were "resistant" to the normally IgE-inducing protein in alum challenge (6). The mean total IgE level in the pDNA-primed was also much lower than in the protein-protein-injected control mice. To evaluate the antigen specificity of the "protective" effect on IgE formation by pCMV-*lacZ* priming on subsequent IgE responses, mice primed with pCMV-*lacZ* were injected with hen's egg albumin (ovalbumin, OVA) in alum. These mice made an IgE anti-OVA response similar to that of a primary OVA in alum injection in control mice, indicating that the "protective" effect on IgE formation by pDNA immunization was antigen specific. The persistence of the predominant Th1 response, even after β -gal in alum injection, in the pCMV-*lacZ*-primed mice was shown by the lymphokine secretion pattern. Spleen cells from pCMV-*lacZ*-primed and β -gal in alum-

boosted mice secreted even more IFN- γ than cells from pDNA-primed control mice and still only very low quantities of IL-4 and IL-5 (6). This indicated that the primary Th1 response induced with pDNA was dominant and prevented a Th2 response to the protein in alum booster injection.

Gene vaccination induces a Th1 response even in the presence of an ongoing Th2 response

In the next set of experiments, we evaluated the effect of pCMV-*lacZ* boosting on an established anti- β -gal Th2 response. As shown in Fig. 1B, boosting of β -gal in alum-primed mice with pCMV-*lacZ* resulted in a fivefold increase of the pre-existing IgG2a antibody level as compared to the protein in saline-boosted control mice, which showed a decrease of the IgG2a antibody level. Moreover, pCMV-*lacZ* boosting resulted in a decrease of about 75% of the IgE antibody levels by 6 weeks after pDNA boosting. The total IgE level also dropped and was about 30% of that of the control group (921 ng/ml vs 3223 ng/ml). Intradermal injection of pDNA encoding OVA (pCMV-OVA) into the β -gal in alum-primed mice had no effect on the IgE antibody levels, indicating that pCMV-*lacZ* boosting specifically lowered the IgE anti- β -gal antibody level. These experiments suggested that the pre-existing Th2 response to β -gal could not prevent the "therapeutic" effect of a secondary immunization with pDNA. This was confirmed by the lymphokine secretion of *in vitro* activated CD4⁺ spleen cells. T cells from β -gal in alum-primed and pCMV-*lacZ*-boosted mice secreted IFN- γ in quantities similar to mice receiving a primary pCMV-*lacZ* injection (6). Furthermore, the pCMV-*lacZ* boosting caused a decrease of the IL-5 secretion by about 60% but had no effect on IL-4 secretion. This observation was subsequently confirmed also in other antigen systems. It appears that it is more difficult to induce a decrease of pre-existing IL-4 than IL-5 secretion by pDNA immunization.

The mechanism of pDNA-induced Th1 responses: the role of immunostimulatory DNA sequences having a CpG motif

Initially, we immunized mice with pDNA constructs that contained the ampicillin resistance gene (*ampR*), which is used to select transfected cells. However, the use of the *ampR* gene is not recommended for pDNA for human use. Therefore, we replaced the *ampR* gene with the kanamycin resistance gene (*kanR*). To our surprise, the *kanR* gene containing plasmids induced only a minimal

immune response to β -gal. The reason for this was the presence of immunostimulatory DNA sequences (ISS) having a CpG motif in the *ampR* but not in the *kanR* gene (9). It had previously been reported that palindromic single-stranded DNA oligodeoxynucleotides (ODN) from mycobacteria having the motif 5'-purine-purine-CpG-pyrimidine-pyrimidine, e.g., AACGTT, activated lymphocyte proliferation and IFN- γ secretion (10, 11). When we inserted one or two of these ISS into the backbone of the *kanR* gene-containing plasmids, they restored the Th1-inducing effect of antigen-pDNA injections (9). We also showed that the ISS ODN induce IFN- α , IFN- β , and IL-12 secretion from antigen-presenting cells (12). Since these three cytokines are all known to induce naive T cells to differentiate into Th1 cells, it is most likely that the presence of ISS in the backbone of plasmids used for gene immunization is the main reason why allergen gene immunization induces a Th1 response, in contrast to the Th2 response elicited by allergens. The fact that gene immunization converts an extracellular antigen into an intracellular antigen may also be an important factor in the mechanism of pDNA-induced Th1 immune responses. The intracellular allergen is probably processed for MHC-class I antigen presentation, and this may cause a much stronger CD8+ T-cell response than exposure to extracellular allergen, and CD8+ cells play an important role in the suppression of IgE antibody formation (13).

The effect of allergen pDNA immunization on the IgE response in mice and rats

Since our initial observation with pDNA immunization employing β -gal or OVA as IgE-inducing antigens in mice, several investigators have confirmed the Th1-inducing effect of allergen pDNA immunization. Slater et al. (14) showed that mice primed with the latex allergen Hev b 5 and boosted with pDNA encoding Hev b 5 had a 23% drop in specific IgE 10 days after pDNA injection. Kim et al. (15) primed mice i.m. with pDNA encoding the house-dust-mite allergen Der p 1 and found IgG2a and IgG2b but only low IgE antibody levels to Der p 1 in such treated mice. Moreover, Hsu et al. (16) immunized rats with pDNA encoding the house-dust-mite allergen Der p 5 and obtained data similar to ours in mice. These authors showed that a pDNA construct encoding Der p 5 induces IgG but no IgE antibodies, and that after challenge with aerosolized Der p 5, the rats showed a decreased histamine release into the bronchoalveolar lavage (BAL) fluid and decreased airway hypersensitivity as compared to control mice immunized with Der

p 5. Similarly, rats injected with pCMV-*lacZ* in our laboratory produced IgG2a and almost no IgE antibodies to β -gal (unpublished data).

Gene vaccination and ISS oligodeoxynucleotides inhibit pulmonary eosinophilic inflammation

To study the effect of allergen gene vaccination on the late-phase allergic reaction in an animal model, mice were primed with three i.d. injections, 2 weeks apart, of 50 μ g each of pCMV-OVA (17). The animals were then boosted with OVA in alum and challenged twice by OVA inhalation. The mice were killed after the last OVA inhalation, and the percent eosinophils was determined in the peripheral blood, the bone marrow, the BAL fluid, and the lung tissue. Priming with pCMV-OVA significantly reduced the percentage of eosinophils in the BAL fluid by 84%, in the lung tissue by 70%, and in the bone marrow by 70% as compared to control animals injected with saline or a nonspecific pDNA (pUC19). These data and the inhibition of histamine release into the BAL fluid of rats immunized with pDNA encoding Der p 5 (16) indicate that allergen pDNA immunization inhibits both immediate and late-phase hypersensitivity in animal models.

Recently, we investigated the direct effect of ISS ODN on eosinophil infiltration in the lung of OVA-sensitized mice (Broide et al., submitted). In these experiments, mice primed with OVA in alum were injected with ISS ODN 6 days before OVA inhalation challenge and the percentages of eosinophils in the lung determined. Injection of ISS ODN before OVA challenge inhibited the eosinophil infiltration in the BAL fluid by 93%, in the lung parenchyma by 91%, in the blood by 86%, and in the bone marrow by 58%. This effect was the result of an ISS ODN-induced decrease in the Th2-type lymphokine secretion (IL-3, IL-5, and GM-CSF) and an increase in Th1-type (IFN- α , - β , and - γ , IL-12) cytokine production in the ISS ODN-treated mice. Similar data were recently reported by Kline et al. (18), who showed that ISS ODN inhibit eosinophilic infiltration of the lung and bronchial hyperreactivity in schistosome egg antigen-sensitized mice after antigen challenge. These data indicate that ISS ODN have not only a stimulatory effect on Th1 cell differentiation but also a suppressive effect on Th2 cell function in allergic inflammation. The precise therapeutic effect of ISS ODN on allergic inflammation remains to be investigated.

Conclusions

The use of classical allergen immunotherapy has greatly decreased over the last 10 years mainly

because the risk-to-benefit ratio is not acceptable at this time. However, if a safe and potent form of immunotherapy could be developed, immunotherapy would probably regain its role as a major treatment for allergies. The recent discovery that allergen gene immunization induces a Th1 anti-allergen response but no IgE antibodies and down-regulates a pre-existing Th2 response suggests that it may be possible to develop a novel and safe form of immunotherapy. Allergen gene immunization would have several advantages over classical immunotherapy:

- 1) Only very small amounts of allergen are secreted by the host's transfected cells, amounts that are unlikely to induce allergic or anaphylactic reactions.
- 2) Allergen gene immunization is likely to have a long-lasting effect since antigen production was shown for over 6 months in the skin in mice after pDNA injection.
- 3) By manipulation of the allergen gene, undesired effects, such as the enzymatic activities of an allergen that could kill transfected allergen-producing cells in the host, could be eliminated.
- 4) pDNA constructs can be made that contain optimal numbers of CpG motif ISS ODN that induce strong Th1 antiallergen responses even in the presence of pre-existing Th2 responses.

All these properties of allergen gene vaccination suggest that it may soon be possible to develop a novel and safe form of immunotherapy that would induce a long-lasting downregulation of pre-existing antiallergen Th2 responses and IgE antibody formation, and, at the same time, induce high levels of protective IgG antibodies.

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