

Progress in the Development of New Methods of Immunotherapy: Potential Application of Immunostimulatory DNA-Conjugated to Allergens for Treatment of Allergic Respiratory Conditions

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Abstract

Allergen immunotherapy was first introduced in the early part of the twentieth century. It is widely practiced despite having specific limitations. Considerable effort has been devoted to developing new modified allergens that, compared with conventional allergen immunotherapy, improve efficacy, decrease the time required to achieve effect, reduce inconvenience, and enhance safety. Increased understanding of allergic respiratory inflammation has led to the development of therapeutic modalities that potentially arrest the disease process in asthma or allergic rhinitis. This paper addresses an adjuvant approach in which highly active immunostimulatory phosphorothioate oligodeoxyribonucleotide sequences (i.e., immunostimulatory DNA) are conjugated to the principal allergenic moiety of a relevant aeroallergen. We have recently completed the first human safety studies in patients with allergic rhinitis with Amb a 1-immunostimulatory oligonucleotide conjugate (AIC) – a novel therapeutic vaccine comprised of Amb a 1, the principal allergenic protein of ragweed, conjugated to specific immunostimulatory oligonucleotides (ISS). The results demonstrate that AIC was several hundred-fold less reactive than a standardized ragweed extract when evaluated by quantitative intradermal skin titration methodology. Furthermore, AIC reduced histamine release from basophils to a similar degree. The DNA vaccine induced IgG antibody production in treated patients. AIC compared with standardized aqueous ragweed

¹ Dr. Creticos is a paid consultant to Dynavax Technologies Corporation. In addition, Dr. Lichtenstein serves on Dynavax's Scientific Advisory Board, and owns Dynavax stock, which is subject to certain restrictions under University policy. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

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exhibited fewer local reactions on skin testing, a finding that suggests that AIC offers the potential for an improved safety profile for immunotherapy. Additional trials to further evaluate the safety, immunologic effect, and therapeutic efficacy of AIC for ragweed-induced allergic rhinitis and asthma are ongoing.

Introduction

Increased understanding of inflammation in patients suffering from allergic rhinitis and/or asthma has led to the development of specific therapeutic modalities that afford the potential to suppress, if not altogether arrest, the disease process. These new molecular approaches include: a) specific therapy with antibodies against immunoglobulin (IgE); b) anticytokine agents – soluble interleukin-4 (IL-4)-receptor antagonists, anti-interleukin-5 (IL-5) antibody; c) DNA vaccination with allergen incorporated into a plasmid; and d) specific T cell-directed therapies such as (i) peptide immunotherapy with T-cell active epitopes; and (ii) adjuvant immunotherapy with immunostimulatory oligonucleotides (e.g., CpG) linked to purified allergen. This paper will deal with the latter of these modalities.

IgE antibody is a critical determinant in the initiation of the allergic reaction. It is found circulating freely and bound to tissue-resident mast cells and circulating basophils (1,2). In the context of the IgE-mediated allergic reaction, the mast cell is regarded as the pivotal cell in initiating the allergic response; while the basophil is involved in development of the persistent (chronic) allergic inflammatory response. These cells elaborate a variety of inflammatory mediators (e.g., histamine, prostaglandin D₂, leukotrienes) and specific cytokines (IL-4, IL-13, TNF α) capable of inducing the subsequent allergic inflammatory cascade (3,4).

Within this context, it is recognized that the T cell is the principal orchestrator of the chronic inflammatory cascade, as this cell elaborates specific cytokines and chemokines responsible for recruitment and/or activation of a variety of secondary inflammatory cells including basophils, eosinophils, neutrophils, epithelial cells, and endothelial cells (5-7). Hence, therapies directed at manipulating the T cell, especially within the framework of the Th2:Th1 paradigm, may more effectively abrogate, or even arrest, the allergic diathesis (8-10).

Conventional immunotherapy is a well-recognized modality that aims to modulate an allergic patient's immune response through administration of increasing doses of an extract, comprised of the aeroallergens to which the patient has been demonstrated to be allergic, and thereby to attenuate or eliminate the patient's symptoms. Allergen immunotherapy was first introduced in the early part of the 20th century and has been widely practiced. Controlled clinical trials have demonstrated the therapeutic efficacy and detailed the favorable immunologic changes associated with allergen immunotherapy for the treatment of allergic rhinitis, asthma, and venom sensitivity (11-15).

This approach has a number of encumbrances including the need for frequent dosing over years – which impacts upon patient compliance; the need to administer a relatively large dose of the immunizing agent to achieve control of symptoms; and the

potential for clinically significant allergic reactions to the treatment. Therefore, considerable effort has been devoted to developing improved immunotherapy for treatment of allergic diseases to a) improve efficacy; b) decrease the time required to achieve effect; c) reduce the inconvenience and hence improve compliance with immunization regimens; and d) enhance safety.

Allergen dose is limited by systemic reactions to the respective immunizing allergens. Thus, efforts have largely been directed at decreasing the "allergenicity" of the antigens (i.e., their potential for inducing an allergic reaction) while maintaining or heightening their immunogenicity (i.e., their ability to generate IgG anti-allergic antibodies which usually parallel the clinical response). Although various chemical modifications of allergens have been attempted, the end-result has been that allergenicity and immunogenicity have either decreased or increased in tandem. A therapeutic material with reduced allergenicity, but maintained immunogenicity, that could be given in a few doses would have important therapeutic implications. Millions of patients with poorly controlled allergic rhinitis and asthma would be candidates for such a form of immunomodulation.

This paper addresses a specific adjuvant approach in which highly active immunostimulatory phosphorothioate oligodeoxyribonucleotide sequences (immunostimulatory DNA) are conjugated to the principal allergenic moiety of a relevant aeroallergen (e.g., ragweed Amb a 1) (16,17) (Figure 1). This adjuvant approach may prove to be highly effective at directing the immune response towards upregulation of a more favorable Th1 phenotypic expression to counterbalance the untoward Th2-driven pro-inflammatory allergic diathesis.

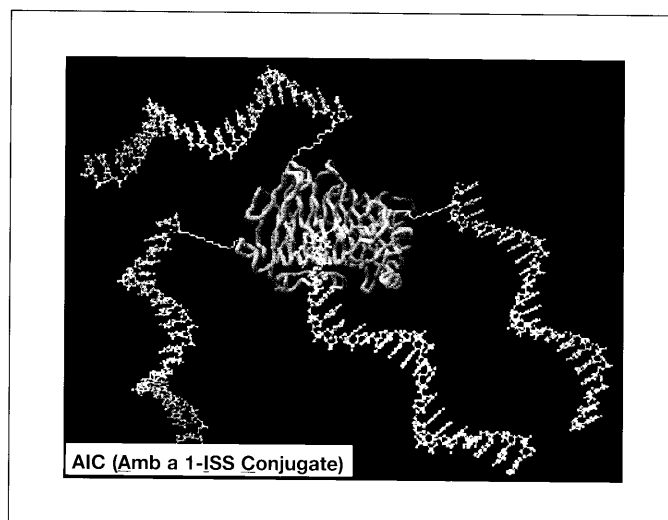


Fig. 1.
AIC (Amb a 1-ISS Conjugate).

The opportunity to present our work on immunostimulatory DNA conjugated to allergen to the Paul-Ehrlich-Symposium is particularly apropos as it provides a forum to highlight the close interaction and positive interplay between/amongst the US FDA regulatory agency, the pharmaceutical company responsible for developing a potential

commercial product, and the regulatory design, and conduct.

Mechanisms of

To improve the clinical pathophysiology of allergic disease in a susceptible individual, the goal is to modulate the cell activation and subsequent release of mediators. In this context, the goal is to modulate the cells that release these molecules. If appropriate, the goal is to modulate the Th2 cells that produce these molecules. This in turn results in a reduction of eosinophils and mast cells, and hence inflammation.

Our allergy group has been studying the mechanism of action of allergen immunotherapy. In a study where both IgE and IgG levels were measured, we found that IgG levels further increased and fail to be blocked by the presence of IgG antibody.

Nasal allergen immunotherapy (NAIT) is a form of chemical modification that parallel the acute clinical response, swelling. Furthermore, we observed (25,26) that the inflammatory mediators released by eosinophil migration.

Nasal biopsies taken after challenge, showed a decrease in gamma, IFN- γ , and an accumulation of Th1 cells. These techniques did not show a decrease in Th2 cells. Mononuclear cells were isolated from Th2 cells and was able to decrease when these cells were exposed to a strable effect on the

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To improve materials for immunotherapy, it is critical to understand the underlying pathophysiology of the allergic disease process. Upon allergen exposure in a susceptible individual, there is an acute allergic response – reflective of IgE-dependent mast cell activation with the resultant release of histamine, leukotrienes, and other chemical mediators. In initiating the allergic reaction, allergen is processed by antigen-presenting cells that display the allergen in association with class II human leukocyte antigen molecules. If appropriate co-stimulatory signals are present, the result is the induction of Th2 cells that produce "pro-inflammatory" cytokines (e.g., IL-4, IL-5, IL-6, IL-10). This in turn results in the recruitment of various inflammatory cells – such as eosinophils and basophils – with the subsequent development of persistent airway inflammation (18-20).

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Our allergy group at Johns Hopkins has examined the mechanisms by which allergen immunotherapy effects clinical improvement. As increasing doses of extract are injected, both IgG- and IgE-specific antibodies increase. With continuing therapy, IgG levels further increase and plateau whereas antigen-specific IgE titers gradually decline and fail to be boosted by subsequent environmental exposure to the allergen. The level of IgG antibody is usually but not always a predictor of clinical success (21-24).

Nasal allergen challenge has proven to be a useful tool to evaluate the effect of immunotherapy. Provocation of the nose with allergen causes the release of a variety of chemical mediators (e.g.: histamine, prostaglandins, and leukotrienes) which parallel the acute clinical manifestations – including sneezing, rhinorrhea, and mucosal swelling. Furthermore, in many allergic patients, a late phase of airway inflammation is observed (25,26). Patients successfully immunized demonstrate less immediate inflammatory mediator release in their nasal secretions and a reduction in late-phase eosinophil migration (27-30).

a 1-ISS Conjugate).

Nasal biopsies of grass-immunized rhinitic patients, that have undergone allergen challenge, show a significant increase in message for specific Th1 cytokines (interferon gamma, IFN- γ) and IL-12. This finding is paralleled by a reduction in allergen-induced accumulation of total numbers of CD4⁺ T cells and eosinophils. Of note, these techniques did not show evidence of down-regulation of the expression of cytokines elaborated from Th2 cells (31,32). However, Secrist et al. (33) cultured peripheral blood mononuclear cells from allergic patients receiving maintenance grass immunotherapy and was able to demonstrate a significant decrease in allergen-induced IL-4 synthesis when these cells were exposed to allergen (*in vitro*). However, in his studies no demonstrable effect on IL-2 or IFN- γ synthesis was observed.

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The problem with current immunotherapy lies in the need to administer a dose large enough to induce the desired changes without causing serious allergic side effects. Towards this goal, Dynavax Technologies Corporation has developed a novel product

consisting of ragweed allergen (Amb a 1) linked to immunostimulatory phosphorothioate oligodeoxyribonucleotide. The Amb a 1-immunostimulatory oligonucleotide conjugate (AIC) induces an enhanced, ragweed-specific Th1-type response in mice, dogs, and primates, in comparison to either Amb a 1 alone or Amb a 1 + alum (16,34).

Background for adjuvant approaches

A variety of molecules including lipopolysaccharides, aluminum hydroxide salts, and Freund's adjuvant have been recognized to possess immunostimulatory properties. Tokunaga and his colleagues made the initial discovery of the adjuvant effect of bacterial DNA (35,36). Subsequent confirmation of the sequence specificity of the immunostimulatory DNA was made by Pisetsky et al. (37) and Klinman et al. (38,39). Krieg et al. (40) recognized that specific oligonucleotides derived from bacterial DNA could induce B-cell activation. Subsequently, Raz, Carson, and colleagues (41,42) made the observation of enhanced immunostimulatory properties if these specific immunostimulatory sequences were incorporated into a plasmid.

The basis for enhancement of Th1 response by immunostimulatory DNA sequences is the recognition that bacteria induce an immune response that is characterized by a potent IL-12 activation of Th1 cells to secrete IFN- γ and a lower level activation of Th2 cells. These properties are related to the higher frequency of CpG motifs and to the absence of cytosine methylation in bacterial as opposed to vertebrate DNA (methylation abolishes the immunostimulatory activity) (45). The effects of bacterial DNAs can be mimicked using synthetic oligonucleotides – allowing a more accurate definition of the specific bacterial immunostimulatory DNA sequences.

Early research efforts identified optimal ISS sequences containing palindromic hexamers based on the general formula of: 5'-purine-purine-CG-pyrimidine-pyrimidine-3' (e.g., 5'-GACGTC-3', 5'-AGCGCT-3' and 5'-AACGTT-3'). Subsequent work has extended these observations to include CpG-enriched non-palindromic oligonucleotides with ISS activity (8,46).

Both bacterial DNA and specific synthetic oligonucleotides (ISS) have multiple effects on the immune system including induction of B cell proliferation and immunoglobulin production; secretion of IFN- α , - β , IL-12, and IL-18 (interferon gamma inducing factor (IGIF)), by macrophages; and IFN- γ secretion from natural killer cells. Such cytokines are involved in the differentiation of Th0 to Th1-type cells upon engagement with specific antigen. Therefore, bacterial DNAs appear to stimulate the innate immune system to produce IFN- γ and specific inducers of IFN- γ (IFN- α , IFN- β , IL-12, IL-18), and foster a cytokine milieu that drives the adaptive immune response towards a Th1 phenotype (8,10).

Effect of Immunostimulatory Oligonucleotide Sequences on Antibody Production

The immunomodulatory effect of conjugation of allergen with ISS is easily demonstrated in a murine animal model in which immunization with a conjugate of Amb a 1

and ISS (AIC) produces a Th1 phenotype that observed with alum. An inhibition of Th2 model IgG2a is also induced through Th1. This also occur in previous opportunity not to employ this therapy.

Effect of ISS on

AIC results in a shift towards a Th1 phenotype. It generated responses in macrophage monocytes. Th1 activation is increased.

Figure 2 demonstrates the model as a result of that would be observed mixed with ISS. Similar observations obtained from murine

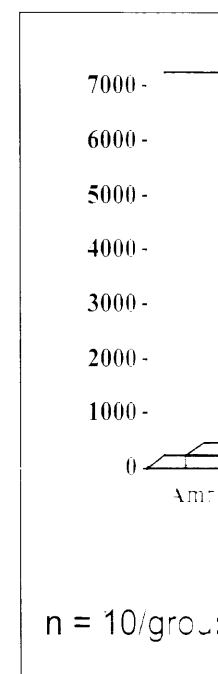


Fig. 2. Activation of

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and ISS (AIC) promotes IgG2a antibody production that is significantly greater than that observed with either allergen mixed with ISS, allergen + alum, or allergen alone. An inhibition of IgE production also occurs. It is also recognized that in the murine model IgG2a is a marker of induction of a Th1 response; whereas IgE production is induced through Th2 mechanisms. These changes in IgG and IgE antibody responses also occur in previously sensitized and not only naïve animals (16,47). This affords the opportunity not only to use this approach as a prophylactic intervention, but also to employ this therapeutic modality in sensitized individuals (48).

Effect of ISS on cellular pathways

AIC results in the preferential induction of naïve CD4⁺ T cells with differentiation towards a Th1 profile. The corollary to this observation, down-regulation in Th2-mediated responses to allergen (e.g., IL-4, IL-5), is thought to be the result of macrophage/monocytic activation (IL-12, IL-18, IFN- α/β) with constituent effects on Th1 activation ($\uparrow$ IFN- γ) (16,17,47).

Figure 2 demonstrates (49) the up-regulation of IFN- γ (Th1 profile) in a murine model as a result of AIC immunization, in contrast to the Th2 cytokine dysregulation that would otherwise be observed with allergen alone, allergen + alum or allergen mixed with ISS (but not linked to this specific adjuvant). Marshall et al. (17) have made similar observations with *in vitro* studies in peripheral blood mononuclear cells obtained from ragweed-allergic human subjects.

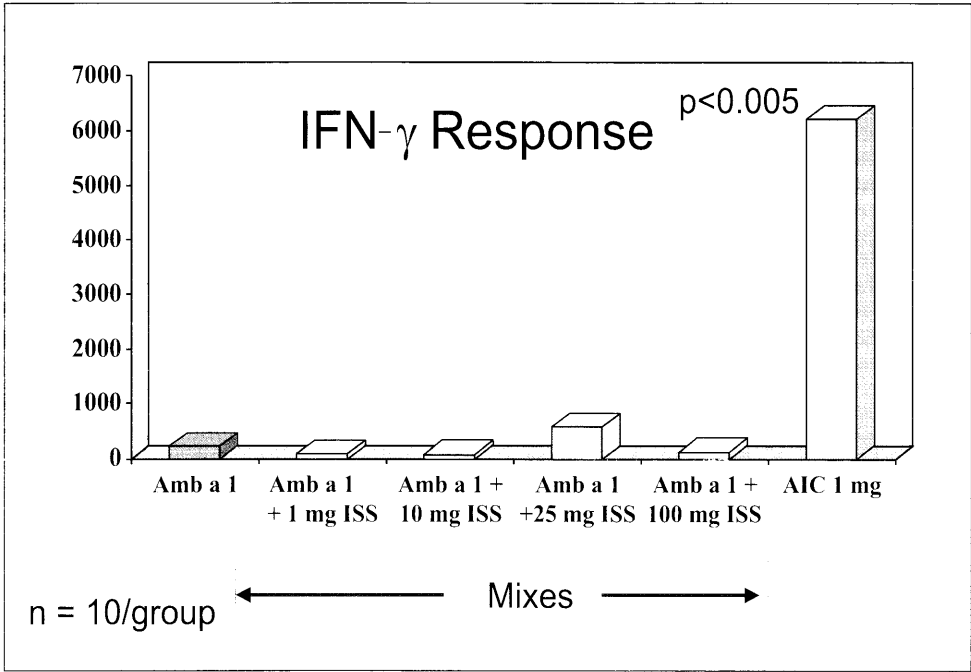


Fig. 2. Activity of AIC vs. Amb a 1/ISS mix in mice.

Clinical applications with DNA vaccine

This paper reviews in a cursory fashion our recently completed initial human safety study with the use of immunostimulatory DNA in ragweed-allergic rhinitic patients. In this U.S. FDA-defined clinical safety study, the technique of quantitative intradermal endpoint skin test titration was used to assess the relative potency of AIC as compared to a standardized ragweed extract. The results of this study demonstrated that AIC was several hundred-fold less reactive than licensed ragweed when evaluated by this parallel line bioassay methodology (50). Histamine release from basophils was similarly reduced in these ragweed-allergic subjects (50). Our observations that AIC exhibited fewer local reactions on skin testing, in comparison to licensed ragweed, suggests that this novel product may offer the potential for an improved safety profile for immunotherapy.

Our initial clinical trial, with subcutaneous administration of the AIC product in a conventional weekly (24-injection) regimen, showed that the vaccine induced IgG antibody production in ragweed allergic patients (51). We have also recently completed a clinical trial with a more favorable 6-injection AIC regimen and will report the promising results of this trial in the near future (52).

References

1. Ishizaka K, Ishizaka T. Human reaginic antibodies and immunoglobulin. *Eur J Allergy* 1968; 42: 333-363.
2. Ishizaka T, Hirata F, Ishizaka K, et al. Stimulation of phospholipid methylation, Ca^{2+} influx and histamine release by bridging of IgE receptors on rat mast cells. *Proc Natl Acad Sci USA* 1980; 77: 1903-1906.
3. Wasserman SI. Mediators of immediate hypersensitivity. *J Allergy Clin Immunol* 1983; 372: 101-115.
4. Lewis RA, Austen KF. Mediation of local homeostasis and inflammation by leukotrienes and other mast cell dependent compounds. *Nature* 1981; 293: 103-108.
5. Kay AB. T Lymphocytes in chronic asthma. In: Marone G, Austen KF, Holgate ST, Kay AB, Lichtenstein LM, eds. *Asthma and Allergic Diseases*. San Diego, CA: Academic Press 1998: 207-221.
6. Cousins DJ, Staynov DZ, Lee TH. Regulation of IL-4, IL-5 and GM-CSF in T lymphocytes. In: Marone G, Austen KF, Holgate ST, Kay AB, Lichtenstein LM, eds. *Asthma and Allergic Diseases: Physiology, Immunopharmacology and Treatment*. San Diego, CA: Academic Press 1998: 193-203.
7. Bochner BS, Schleimer RP. Endothelial cells and cell adhesions. *Allergy* 1997; 2: 251-276.
8. Van Uden JV, Raz E. Immunostimulatory DNA and applications to allergic disease. *J Allergy Clin Immunol* 1999; 104: 902-910.
9. Broide D, Schwarze J, Tighe H, et al. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J Immunol* 1998; 161: 7054-7062.
10. Horner AA, Van Uden JH, Zubeldia JM, et al. DNA-based immunotherapeutics for the treatment of allergic disease. *Immunol Rev* 2002; 79: 102-118.
11. Creticos PS. Immunologic changes associated with immunotherapy. *Immunotherapy of IgE-mediated disorders*. *Immunol Allergy Clin North Am* 1992; 12: 13-37.
12. Creticos PS. Immunotherapy. In: Rich RR, Shearer WT, eds. *Clinical Immunology. Principles and Practice*. St. Louis, Missouri: Mosby 1995: 2002-2018.
13. Bush K, Ritter M. Allergen immunotherapy for the patient with allergic rhinitis. *Immunol Allergy Clin North Am* 1992; 12: 107-124.
14. Creticos PS. The consideration of immunotherapy in the treatment of allergic asthma. *J Allergy Clin Immunol* 2000; 2: S559-S574.
15. Creticos PS, Reed CE, Norman PS, et al. Ragweed immunotherapy in adult asthma. *N Engl J Med* 1996; 334: 501-506.
16. Tighe H. Takaragweed allergen. *Immunol* 2000.
17. Marshall JD. Allergen promotes T_H1 from human cells.
18. O'Hehir R. Ragweed allergen. *Ann F*
19. Robinson D. mRNA expression challenge in ragweed.
20. Hamid QA. Suppression of allergic response. *Clin Immun*
21. Lichtenstein LM. Relationship to asthma.
22. Creticos PS. Immunotherapy.
23. Creticos PS. Allergic rhinitis and ragweed immunotherapy.
24. Haugaard B. Standardized ragweed extract. *Clin Immun*
25. Naclerio RM. *Engl J Med* 1996.
26. Creticos PS. Allergic patients' sensitivity.
27. Creticos PS. Allergic fever patients' sensitivity.
28. Creticos PS. Allergic immunotherapy.
29. Iliopoulos D. Allergic challenge nasal spray. *Allergy Clin Immun*
30. Furin MJ. Neutrophil migration and asthma.
31. Varney VA. Allergic reaction and asthma. *Invest* 1993; 42.
32. Durham SR. Selective intranasal administration of CD4⁺ expressing mast cells.
33. Secrist H. Allergic CD4⁺ T cells.
34. Tighe H. Tumor necrosis results in a new. *Immunol* 2000.
35. Tokunaga T. Allergic from *M. tuberculosis* sensitivity. *J Natl Cancer*
36. Yamamoto S. Allergic ferons, activated.
37. Pisetsky DS. Allergic
38. Klinman DM. Allergic phocytes. *Immunol* 1996; 93: 287-292.
39. Klinman DM. Allergic of DNA vaccine.

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16. Tighe H, Takabayashi K, Schwartz D, et al. Conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol* 2000; 105: 124-134.
17. Marshall JD, Abtahi S, Eiden JJ, et al. Immunostimulatory sequence DNA linked the Amb a 1 allergen promotes TH 1 cytokine expression while down regulating TH 2 cytokine expression in PBMC's from human patients with ragweed allergy. *J Allergy Clin Immunol* 2001; 108: 191-197.
18. O' Hehir R, Garman R, Greenstein J, et al. The specificity and regulation of T cell responsiveness to allergen. *Ann Rev Immunol* 1991; 9: 676-695.
19. Robinson D, Hamid Q, Bentley A, et al. Activation of CD4⁺ T cells increased TH 2-type cytokine mRNA expression and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J Allergy Clin Immunol* 1993; 92: 3123-3124.
20. Hamid QA, Schotman E, Jacobson, et al. Increases in IL-12 messenger RNA⁺ cells accompany inhibition of allergen-induced late skin responses after successful grass pollen immunotherapy. *J Allergy Clin Immunol* 1997; 99: 254-260.
21. Lichtenstein LM, Ishizaka K, Norman PS, et al. IgE antibody measurements in ragweed hay fever: relationship to clinical severity and the results of immunotherapy. *J Clin Invest* 1973; 52: 472-482.
22. Creticos PS. Immunotherapy with allergens. *JAMA* 1997; 268: 2834-2839.
23. Creticos PS, Van Metre TE Jr, Mardiney MR, et al. Dose response of IgE and IgG antibodies during ragweed immunotherapy. *J Allergy Clin Immunol* 1984; 73: 94-104.
24. Haugaard L, Dahl R, Jacobsen L, et al. A controlled dose-response study of immunotherapy with standardized, partially purified extract of house dust mite: clinical efficacy and side effects. *J Allergy Clin Immunol* 1993; 91: 709-722.
25. Naclerio RM, Proud D, Togias AG, et al. Inflammatory mediators in late antigen-induced rhinitis. *N Engl J Med* 1985; 313: 65-70.
26. Creticos PS, Peters SP, Adkinson, NF Jr, et al. Peptide leukotriene release after antigen challenge in patients sensitive to ragweed. *N Engl J Med* 1984; 310: 1626-1630.
27. Creticos PS, Adkinson NF Jr, Kagey-Sobotka A, et al. Nasal challenge with ragweed pollen in hay fever patients: effect of immunotherapy. *J Clin Invest* 1985; 76: 2247-2253.
28. Creticos PS, Marsh DG, Proud D, et al. Responses to ragweed pollen nasal challenge before and after immunotherapy. *J Allergy Clin Immunol* 1989; 84: 197-205.
29. Iliopolous O, Proud D, Adkinson NF Jr, et al. Effects of immunotherapy on the early, late and rechallenger nasal reaction to provocation with allergen: changes in inflammatory mediators and cells. *J Allergy Clin Immunol* 1991; 87: 855-866.
30. Furin MJ, Norman PS, Creticos PS, et al. Immunotherapy decreases antigen-induced eosinophils migration into the nasal cavity. *J Allergy Clin Immunol* 1991; 88: 27-32.
31. Varney VA, Hamid QA, Gaga M, et al. Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. *J Clin Invest* 1993; 92: 644-651.
32. Durham SR, Sun Ying, Varney VA, et al. Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4⁺ T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon- α . *J Allergy Clin Immunol* 1996; 97: 1356-1365.
33. Secrist H, Chelen CJ, Yan W, et al. Allergen immunotherapy decreases interleukin 4 production in CD4⁺ T cells from allergic individuals. *J Exp Med* 1993; 178: 2123-2130.
34. Tighe H, Takabayashi K, Schwartz D, et al. Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. *Eur J Immunol* 2000; 30: 1939-1947.
35. Tokunaga T, Yamamoto H, Shimada S, et al. Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG: I: isolation, physicochemical characterization, and antitumor activity. *J Natl Cancer Inst* 1981; 72: 955-962.
36. Yamamoto S, Yamamoto T, Shimada S, et al. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. *Microbiol Immunol* 1992; 36: 983-997.
37. Pissetsky DS. Immune activation by bacterial DNA: a new genetic code. *Immunity* 1996; 5: 303-310.
38. Klinman DM, Yi AK, Beaucage SL, et al. CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci USA* 1996; 93: 2879-2883.
39. Klinman DM, Yamshchikov G, Ishigatsubo, Y. Contributions of CpG motifs to the immunogenicity of DNA vaccines. *J Immunol* 1980; 158: 3635-39.

40. Krieg AM, Yi Ak, Matson S, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995; 374: 546-549.
41. Raz E, Carson DA, Parker SE, et al. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc Natl Acad Sci USA* 1994; 91: 9519-9523.
42. Raz E, Tighe H, Sato Y, et al. Preferential induction of a TH 1 immune response and inhibition of specific IgE anti-body formation by plasmid DNA immunization. *Proc Natl Acad Sci USA* 1996; 93: 5141-5145.
43. Halpern MD, Kurlander RJ, Pisetsky DS. Bacterial DNA induces murine interferon-gamma production by stimulation of interleukin-12 and tumor necrosis factor-alpha. *Cell Immunol* 1996; 167: 72-78.
44. Yamamoto S, Yamamoto T, Kataoka T, et al. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J Immunol* 1992; 148: 4072-4076.
45. Bird AP. DNA methylation and the frequency of CpG in animal DNA. *Nucleic Acids Res* 1980; 8: 1499-1504.
46. Yamamoto T, Yamamoto S, Kataoka T, et al. Synthetic oligonucleotides with certain palindromes stimulate interferon production of human peripheral blood lymphocytes *in vitro*. *Jpn J Cancer Res* 1994; 85: 775-779.
47. VanNest G, Eiden JJ, Tuck SF, et al. Conjugation of immunostimulatory DNA (ISS) to the major short ragweed allergen, Amb a 1, enhances immunogenicity and reduces allergenicity. *J Allergy Clin Immunol* 2000; 105: S70.
48. Horner AA, Takabayashi K, Beck L, et al. Optimized Conjugations Ratios Lead to Allergen Immunostimulatory Oligodeoxyribonucleotide Conjugates with Retained Immunogenicity and Minimal Anaphylactogenicity. *J Allergy Clin Immunol* 2002; 110: 413-420.
49. Creticos PS. New Methods of Immunotherapy in Asthma and Allergic Rhinitis. *Advanced Studies in Medicine* 2000; 2 (7): 270-275.
50. Creticos PS, Eiden JJ, Balcer SL, et al. Immunostimulatory oligonucleotides conjugated to Amb a 1: Safety, skin test reactivity, and basophil histamine release. *J Allergy Clin Immunol* 2000; 105: S70.
51. Creticos PS, Balcer SL, Schroeder JT, et al. Initial immunotherapy trial to explore the safety, tolerability, and immunogenicity of subcutaneous injections of an Amb a 1 immunostimulatory oligonucleotide conjugate in ragweed-allergic adults. *J Allergy Clin Immunol* 2001; 107: 216. *Advanced Studies in Medicine* 275.
52. Creticos PS, Eiden JJ, Broide D H, Balcer-Whaley, SL, Schroeder, JT, Khattignavong, A, Li H, Norman P, Hamilton, R. Immunotherapy with Immunostimulatory Oligonucleotides Linked to Purified Ragweed Amb a 1 Allergen: Effects on Antibody Production, Nasal Allergen Provocation, and Ragweed Seasonal Rhinitis. *J. Allergy Clin. Immunol* 2002; 109, No 4: 743-44 (3)

Discussion

Drachenberg: What is the difference between the ISS approach and the CpG approach?

Creticos: That is a good question. As you know, on the one hand Art Creagan and his colleagues have now formed a company and make just the ISS by itself using it as an adjuvant to add to an allergen mix as an example. It is a simple adjuvant. In this approach these oligonucleotides are covalently linked to the allergen of interest. The question therefore becomes: Is one approach superior or not? In terms of the animal studies that were done by Eyal Raz, they looked at the ISS by itself and then looked at the ISS conjugated to the purified Amb a 1 from ragweed. They were able to show in animal studies a markedly better response in terms of Th1 activity with production of interferon- γ and suppression or non-induction of IL-4 and IL-5. So, in their hands they felt that conjugating the product provided a superior method of immunization. But it may be possible as we have seen to tinker with and adjust the ISS in a way that you might be able to show comparable responses without having to use the vehicle of attaching it to an allergen. But right now, the state-of-the-art would say that in the animal

models we see a... supplied Jason M... studies of periph... strated exactly wh... of interferon- γ , b...

Schneider: My... separate compon... simply the recom... ment you would h... and the conjugate...

Creticos: Maybe... this (what?) to ev... planned subsequen... immunized last s... which would have... followed them ag... antibody and T ce... induction of toler... and post immun... post during the s... dose. Because my... this weekend as... 15 μ g that I have... a markedly better... That might then... then, allows us to... whether that be... timate question... shown, for exampl... cific allergen to w... gone up to 36 and... think it is possibl... have got a produc... to be safer. If so... say that we have...