Amb a 1-immunostimulatory
Oligodeoxynucleotide Conjugate
Immunotherapy Increases CD4+CD25+
T Cells in the Nasal Mucosa of
Subjects with Allergic Rhinitis
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ABSTRACT
Background: We have previously shown that short-course treatment with Amb a 1-immunostimulatory phosphorothioate oligonucleotide conjugate (AIC) before the ragweed season modifies the response of the nasal mucosa to allergen challenge in ragweed-sensitive patients by increasing Th1 cytokines and decreasing both Th2 cytokines and eosinophilia after the ragweed season. The effect of AIC immunotherapy on CD4+CD25+ T cell expression in the nasal mucosa is unknown.
Objective: To determine the in vivo effect of short-course AIC immunotherapy on CD4+CD25+ regulatory T cells in the nasal mucosa of ragweed-sensitive subjects.
Methods: 19 ragweed-sensitive patients with allergic rhinitis were randomly assigned to receive either 6 escalating doses of AIC (0.06–12 μg; n = 12) or placebo (n = 7) at weekly intervals immediately before the 2001 ragweed season. Nasal biopsies were taken at baseline prior to immunization and again post immunization 24 hours after ragweed allergen challenge at the start and end of the ragweed season. The expression of T regulatory cells, IL-10 and TGF-β was assessed at each time point by immunohistochemistry.
Results: The numbers of allergen-induced CD4+-, CD4+CD25+-, IL-10- and TGF-β-positive cells in the nasal mucosa after AIC immunization before the ragweed season did not differ between the two groups. Repeat challenge after the ragweed season led to a significant increase in CD4+CD25+ cells in AIC-compared with placebo-treated subjects. A trend toward an increase in IL-10-positive cells in the AIC-treated group did not reach statistical significance.
Conclusions: Short-course AIC immunotherapy increases CD4+CD25+ regulatory T cell infiltration in the nasal mucosa following allergen challenge after seasonal ragweed-pollen exposure.

KEY WORDS
allergic rhinitis, Amb a 1-immunostimulatory oligodeoxynucleotide, immunotherapy, ragweed-pollen allergy, regulatory T cell

ABBREVIATIONS
AIC, Amb a 1-immunostimulatory phosphorothioate oligonucleotide conjugate; ISS, immunostimulatory DNA sequences; TLR, toll-like receptor; PAMP, pathogen associated molecular pattern; pDC, plasmacytoid dendritic cells; TGF-β, transforming growth factor beta; APAAP, alkaline phosphatase-anti-alkaline phosphatase
INTRODUCTION
A Th2-dominant immune imbalance exists in the immune response of allergic patients and this is thought to be a main target for intervention. Allergen immunotherapy represents a therapeutic option for patients with IgE-mediated allergic rhinitis whose seasonal symptoms are not adequately controlled by allergen avoidance and symptomatic pharmacological treatments. Allergen-specific immunotherapy aims to modify this immune imbalance particularly by attempting to redirect the established Th2-dominated response toward more balanced Th1/Th2 responses. Considerable attention has been focused on the development of more novel immunotherapies that are safer and have shorter treatment regimens than conventional allergen-specific immunotherapy.

The use of toll-like receptor (TLR) ligands in combination with allergens to modify the function of antigen presenting cells has been proposed as a new strategy for biasing the immune response toward Th1 in allergic disease. Amb a 1-immunostimulatory phosphorothioate oligonucleotide conjugate (AIC) one such novel compound, has received a lot of attention in recent years and has lately undergone large multicenter phase 2/3 clinical trials with encouraging short- and long-term clinical results in subjects with moderate-to-severe ragweed allergy. It is produced by covalently linking the purified short ragweed pollen allergen, Amb a 1 conjugate, to a phosphorothioate oligodeoxyribonucleotide immunostimulatory sequence (ISS-ODN) containing a CpG motif. The ISS is a short synthetic DNA sequence that binds to TLR9 on plasmacytoid dendritic cells (pDC) with subsequent activation of the innate immune system. Due to the enhanced immunogenicity and reduced allergenic properties of AIC compared with Amb a 1 alone, this vaccine has potential as a novel form of immunotherapy with a shorter treatment regimen and higher doses for subjects with allergy to ragweed pollen.

The mechanisms by which AIC modulates the immune response are currently the focus of many studies. We have previously reported that short-course AIC immunotherapy before the ragweed season modifies the response of the nasal mucosa to allergen challenge in ragweed-sensitive patients and causes a significant increase in the number of IFN-γ mRNA-positive cells with concomitant decreases in both IL-4 mRNA-positive cells and eosinophilia in nasal biopsies of ragweed-sensitive patients after the ragweed season compared with placebo-treated patients. Siemons et al. demonstrated a similar biasing of the immune response towards Th1 in primary peripheral blood mononuclear cells from patients with ragweed allergy cultured for a short time with AIC.

Suppression of the Th2 response by AIC therapy may occur through the induction of a subtype of T cell with immunosuppressive functions called regulatory T cells. These CD4+CD25+ T-cells with regulatory function have a specific cytokine profile producing both IL-10 and TGF-β and are known to suppress inflammation in both normal and atopic patients. Conventional allergen-specific immunotherapy induces a population of IL-10-producing CD4+CD25+ regulatory T cells in the peripheral blood of atopic patients following in vitro allergen restimulation. Recently, it has been demonstrated that CpG-activated human pDCs induce CD4+CD25+FoxP3+ regulatory T cells that suppress antigen-stimulated T-cell proliferation in vitro. However, the in vivo effect of AIC immunotherapy on CD4+CD25+ T regulatory cell expression in the nasal mucosa is currently unknown.

The aim of this study was to evaluate the presence of CD4+CD25+ regulatory T cells in the nasal mucosa of ragweed-sensitive patients with allergic rhinitis before and after short-course AIC immunotherapy and to also assess the expression of their suppressive cytokines, IL-10 and TGF-β, in these tissues.

METHODS
SUBJECT RECRUITMENT
19 subjects with known ragweed allergy and a history of symptomatic seasonal rhino-conjunctivitis who gave their written informed consent to have nasal biopsies taken at 3 different time points were included. Many of these subjects demonstrated at least one positive skin prick test response to other allergens suggesting that they were not monosensitized to ragweed. 12 subjects received AIC and 7 age- and sex-matched subjects received placebo. All patients were immunotherapy naïve prior to enrollment. These subjects represent a subgroup of a previous larger study. The study was reviewed and approved by IRB Services (Toronto) and the Comité d’évaluation Scientifique et d’éthique de la Recherche, Hôpital Saint-Luc (CHUM).

STUDY DESIGN SAMPLE COLLECTION
A baseline nasal biopsy was performed on all 19 participating subjects without prior allergen challenge or immunization. Ragweed sensitivity was confirmed in all subjects by nasal challenge with ragweed antigen extract as previously described. Subjects were randomized in an observer-blinded fashion to receive either 6 escalating doses of AIC (0.06, 0.3, 1.2, 3.0, 6.0, and 12.0 μg; n = 12) or placebo (n = 7) by subcutaneous injection at weekly intervals ending 2 to 4 weeks before the 2001 ragweed season. AIC was prepared as previously published. A second nasal biopsy was taken 24 hours after ragweed allergen challenge at the start of the ragweed season and a third biopsy was taken 1 to 2 months after the end of the ragweed season.
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Fig. 1 Graphs showing the number of cells that immunostained positively for CD4⁺CD25⁺ in nasal biopsies of AIC-treated and placebo-treated subjects with allergic rhinitis at baseline, before and after the allergen season.

NASAL CHALLENGE AND BIOPSY
Prior to the initiation of immunotherapy, all subjects underwent a nasal challenge with ragweed antigen extract (3.59 AU; Greer Laboratories, Lenoir, NC). This was performed by applying a filter paper disk soaked in allergen or diluent (0.4% phenol preserved saline) and applied unilaterally to the inferior turbinate for 60 seconds, as previously described. A positive response to ragweed was defined as 3 or more sneezes than experienced with diluent challenge. Patients consenting to serial biopsies were challenged at 24 hours before the biopsy at the time points of 2 weeks after the last injection of AIC or placebo and 1 to 2 months after the end of the 2001 ragweed season as described above. Nasal biopsy specimens measured 2 mm in diameter and were taken from the posterior portion of the anterior third of the inferior turbinate of the nasal mucosa. Alternate nostrils were used for subsequent biopsies. Biopsy specimens were immediately mounted in OCT compound (VWR; Ville Mont-Royal, Quebec, Canada), snap-frozen by means of immersion in isopentane, precooled in liquid nitrogen and stored at −80°C until processing.

IMMUNOHISTOCHEMISTRY
The expression of T regulatory cells, CD4, IL-10 and TGF-β was assessed at each time point by immunohistochemistry on 5-μm cryostat sections fixed in 4% paraformaldehyde. Double staining for CD4 and CD25 was used to identify CD4⁺CD25⁺ regulatory T cells. Briefly, tissue sections were incubated with the appropriate primary antibody or isotype-matched control. Slides were washed in TBS and incubated with secondary antibody. The peroxidase method was employed to immunostain for CD25 which was developed with diaminobenzidine-chromogen (Dako Canada Inc., Mississauga, Ontario, Canada). The alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique was used for all other immunostaining. Counterstaining was performed with haematoxylin.

Immunoreactive positive cells were counted in the submucosa by two blinded independent observers using an Olympus light microscope (Carson Group Inc., Markham, Ontario, Canada) at 200× magnification with an eyepiece graticule of 0.202 mm². The average number of positive cells was calculated from 2 to 4 random, non-overlapping grids and expressed as the mean number of positive cells per millimeter square.

STATISTICS
Results are expressed as means ± SEMs. Statistical analyses were performed using non-parametric tests.
Comparisons between treatment groups were made using the Fisher exact test for proportions and the Wilcoxon 2-sample test for continuous variables. Comparisons within treatment groups were made using the Friedman test followed by the Wilcoxon signed-rank test. All statistical tests were 2-tailed and a P value of <0.05 was considered to indicate a statistically significant difference.

RESULTS
19 subjects fulfilled the criteria and consented to having nasal biopsies taken at all 3 time points. 12 subjects were randomized to receive AIC and 7 age- and sex-matched subjects received placebo.

The numbers of allergen-induced CD4+-, CD4+CD25+-, IL-10- and TGF-β-positive cells present in the nasal mucosa after AIC immunization and before the ragweed season did not differ from the placebo-treated group at the same time point or from baseline numbers. Following repeat challenge after the end of the ragweed season, AIC-treated patients showed a significant increase in CD4+CD25+ cells compared with placebo-treated patients (p < 0.01) and compared with baseline nasal biopsies (p < 0.05) (Fig. 1). A trend toward an increase in IL-10-positive cells in the AIC-treated group post-season compared with both placebo-treated and baseline biopsies did not reach statistical significance (p = 0.08, Fig. 2B). A similar trend was noted for TGF-β in the AIC-treated group (p = 0.09, Fig. 3). There was no difference noted in CD4+ cell numbers in the nasal biopsies taken at the start and end of the ragweed season in either treatment group (Fig. 4).

DISCUSSION
AIC immunotherapy ensures that both ISS and Amb a 1 antigen are presented simultaneously to the same immune cells, producing a highly specific and potent inhibitory effect and thereby suppressing the Th2 cells responsible for the allergic inflammation of ragweed allergy. CpG ODNs stimulate cells that express TLR9, thereby initiating an immunomodulatory cascade that culminates in the production of Th1 and pro-inflammatory cytokines and chemokines. In addition, they improve the antigen-presenting function of DCs, monocytes and macrophages, recruit T cells to the site of ODN administration and induce B cell proliferation.

The generation of allergen-specific regulatory T cells and increased production of their suppressive cytokines, IL-10 and TGF-β, are essential early events in allergen specific immunotherapy. IL-10 is a potent suppressor of both total and allergen-specific IgE and simultaneously increases IgG4 production.
ultimately leads to a decrease in IgE-mediated histamine release. In addition, IL-10 reduces pro-inflammatory cytokine release from mast cells, down-regulates eosinophil function and activity and suppresses IL-5 production by Th0 and Th2 cells.14

In our previous report which includes the same subjects as in the present study, we demonstrated that AIC treatment reduced mRNA expression of IL-4 and IL-5 after the ragweed season.6 These phenomena might be mediated by increase in CD4+CD25+ cells via inhibition of proliferation and cytokine production on Th1 and Th2.9 In contrast, mRNA expression of INF-γ was increased in our previous report.6 However, the effects of CD4+CD25+ cells on expression of INF-γ are different depending on the report.9,11 Increase in CD4+CD25+ cells and its derived IL-10 may be involved in the anti-inflammatory effects of AIC immunization. However, further studies are needed to clarify these mechanisms.

Macrophages and B cells also have the potential to express IL-10 following AIC immunotherapy. IL-10 produced by macrophages is a key modulator of immune tolerance in immunotherapy. In our study, the IL-10-positive cells were morphologically mononuclear cells. However, we did not perform IL-10-positive cells via inhibition of proliferation and cytokine production on Th0 and Th2 cells.9

In this study we have shown for the first time that AIC, a CpG-conjugate allergen immunotherapy, increases CD4+CD25+ T-cell infiltration of the nasal mucosa following allergen challenge. We have also shown a strong trend toward an increase in IL-10 positive cells in the nasal mucosa following allergen challenge.

Cpg vaccines that target TLR9 show encouraging results to date in the treatment of allergic disease. We have shown that AIC immunotherapy modifies the immune response in the nasal mucosa following allergen challenge after seasonal ragweed-pollen exposure via the induction of T regulatory cells. Further experiments aimed at clarifying the exact mechanism of action of these compounds are required.

**ACKNOWLEDGEMENTS**

This study was funded by Dynavax and the Canadian Institute of Health Research (CIHR).

**REFERENCES**


