━━ ORIGINAL ARTICLE ━━

Local Induction of a Specific Th1 Immune Response by Allergen Linked Immunostimulatory DNA in the Nasal Explants of Ragweed-Allergic Subjects

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ABSTRACT

Background: Allergen immunotherapy is effective in allergic individuals however efforts are being made to improve its safety, convenience, and efficacy. It has recently been demonstrated that allergen-linked immunostimulatory DNA (ISS) is effective in stimulating an allergen-specific Th1 response with decreased allergenicity. The objective of this study is to investigate whether ISS linked to purified ragweed allergen Amb-a-1 (AIC) can inhibit local allergen-specific Th2 and induce allergen-specific Th1 responses in explanted nasal mucosa of ragweed-sensitive subjects. In addition, we set out to determine whether AIC is more effective compared to stimulation with unlinked Amb a 1 and ISS.

Methods: Tissue from ragweed-sensitive patients (*n* = 12) was cultured with whole ragweed allergen (RW), Amb-a-1, AIC, Amb-a-1 and ISS (unlinked), or tetanus toxoid (TT) for 24 hours. IL-4, -5, -13, TNF-α and IFN-γ mRNA-positive cells were visualized by *in situ* hybridization and T cells, B cells and neutrophils were enumerated using immunocytochemistry.

Results: RW or Amb-a-1 increased the number of IL-4, IL-5, and IL-13 mRNA+ cells in the tissue compared to medium alone. AIC had similar cytokine mRNA reactivity as control tissue. AIC and TT increased IFNγ-mRNA expression. Unlinked Amb-a-1 and ISS showed similar effects to AIC, however this response was weaker. The number of TNF mRNA+ cells, T cells, B cells and neutrophils remained unchanged.

Conclusions: AIC is effective in stimulating a local allergen-specific Th1- and abolishing Th2-cytokine mRNA reactivity in the nose and may be considered as a strong candidate for an improved approach to immunotherapy in ragweed-sensitive individuals.

KEY WORDS

allergen, allergic rhinitis, CpG motif, immunotherapy, vaccination

ABBREVIATIONS

Amb a 1, short, purified ragweed allergen; AIC, Amb a 1-immunostimulatory phosphorothioate oligonucleotide conjugate; APAAP, alkaline phosphatase anti-alkaline phosphatase; ICC, immunocytochemistry; IL-, interleukin-; ISH, *in situ* hybridization; ISS, immunostimulatory DNA sequences; RW, ragweed; TT, tetanus toxoid.

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INTRODUCTION

Immunotherapy by injection of aqueous allergen extracts is currently used as a form of treatment for allergic diseases. This form of therapy is thought to promote Th1 immunity in allergic patients leading to the alleviation of symptoms that result from allergen-specific Th2 responses. Although generally effective, allergen immunotherapy is associated with a number of disadvantages such as the inconvenience of frequent dosing, its long duration of therapy, as well as the risk of anaphylaxis.[1,2] As a result, many modifications of allergen immunotherapy have been attempted to improve its safety and effectiveness, including the use of allergen extracts. Although chemical modification of allergens can reduce their allergenicity, this strategy can also reduce the allergen's potential for stimulating the immune system, rendering them unsuitable for immunotherapy.[3,4]

Recently, immunostimulatory DNA sequences (ISS) containing CpG motifs have become attractive candidates as they have been shown to act as strong Th1 response-inducing adjuvants.[5] These vaccines target TLR9 on plasmacytoid dendritic cells (pDC) with subsequent activation of the innate immune system.[6] These immune responses favor the development of a Th1 type response. Using a mouse model of eosinophilic airway inflammation, Broide and colleagues have shown that administration of ISS-ODN to mice inhibits both airway hyperresponsiveness and airway eosinophilia by exerting significant inhibitory effects on IL-5, GM-CSF and IL-13 cytokine production whilst inducing the expression of IFN-γ.[7] Direct conjugation of CpG to allergen reduced the dose of CpG required for this response, and has been shown to be effective at modifying Th2 responses in a variety of species.[6]

One compound that has received a lot of attention recently is Amb a 1-immunostimulatory-phosphorothioate oligonucleaotide conjugate (AIC). Conjugation of ISS is on the major short ragweed allergen Amb-a -1, and Tighe and colleagues have demonstrated enhanced immunogenicity and reduced allergenicity in a mouse model of allergic disease and induction of IFN-γ dependent IgG2α and IgG2β antibody production.[6] Although they have further shown unlinked Amb-a-1 with ISS-ODN also induces a Th1 response in mice, this response was significantly weaker than that induced with the allergen-linked-ISS conjugate.[6] Importantly, AIC has also been reported to be less reactive with human IgE anti-Amb a 1 antibodies and to require 30-fold more conjugate than Amb a 1 alone to release the same amount of histamine from basophils of ragweed-sensitive patients.[8] More recently, the effect of AIC was investigated on human cellular immune function where AIC was shown to promote Th1- and down-regulate ragweed-specific Th2-cytokine expression in PBMCs from ragweed-sensitized individuals.[9,10] We have shown in a clinical setting, that short-course immunotherapy with AIC can modify the response of nasal mucosa to allergen challenge in rhinitis patients.[11] This modification was not immediate but was observed 4-5 months after completion of immunotherapy and after seasonal ragweed-pollen exposure. In that study, clinical efficacy was observed in the second ragweed season and without any additional AIC immunizations.

In this follow-on study we set out to investigate the effect of Amb a 1-linked-ISS (AIC) on *ex vivo* ragweed-induced allergic responses in explanted nasal tissue from ragweed-sensitive individuals to investigate the mechanisms which may be involved in AIC's ability to deviate the nasal immune response from Th2- to Th1-phenotype. The unique nature of our explant system allows us to examine the effect of AIC on local, allergen-driven immune responses in the nose and to examine the contribution of local inflammatory cells in this model. In addition, we set out to determine whether AIC is more effective at modulating the allergen-specific local Th1/Th2 responses than stimulation with unlinked Amb-a-1 and ISS.

METHODS

PATIENT SELECTION

Twelve (*n* = 12) patients with allergic rhinitis were recruited from the nasal clinic at the Nôtre Dame Hospital in Montreal, Canada. Hospital ethics committee approval was obtained, and all volunteers gave a written informed consent before commencement of the study. All experiments were carried out in accordance with the principles embodied in the Declaration of Helsinki of 1995. Inclusion criteria included a history of seasonal rhinitis for at least 2 years prior to the study and a positive skin prick test response (>5 mm wheal) to ragweed extract. Patients were excluded if they had perennial allergies, had previously received immunotherapy, or were taking oral anti-inflammatory medication. Additional exclusion criteria included hypogammaglobulinemia, a history of cystic fibrosis, bronchiectasis, immotile cilia syndrome, systemic granulomatous disease, tobacco use, cocaine use, or immunodeficiency. Wherever necessary, antibiotics were withheld for a few weeks before the study.

AIC was prepared by linking purified Amb a 1 with 1018 ISS (sequence: 5'-TGACTGTGAACGTTCGAGA TGA-3'). Cross-linking was done with the heterobifunctional linker sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, which creates stable amide and thioether bonds between the ISS oligonucleotide and the Amb a 1. The average molar ratio of Amb a 1 to 1018 ISS is 1 : 4. A more detailed description of AIC production and characterization has been previously published.[6]

TISSUE CULTURE

Nasal mucosal tissue was resected from patients undergoing sinus surgery, who had given informed consent prior to surgery. The tissue was obtained from the inferior turbinate of ragweed-sensitive individuals with seasonal allergic rhinitis, outside the ragweed season. Serial sections of tissue were placed on 0.4-μm well inserts (Millipore, Bedford, MA, USA) in 2 ml of defined medium and cultured with a) whole ragweed allergen (RW; 50 μg/ml), b) Amb a 1 (20 μg/ml), c) Amb a 1-linked-ISS (AIC, 5μg/ml), d) Amb-a-1 and ISS (unlinked, 20 μg/ml), or e) tetanus toxoid (TT; 0.5 Limes flocculation [Lf]/ml). Cultures were incubated for 24 hours in 5% CO_2/95% air. After this period, tissue was fixed in 4% paraformaldehyde, washed 3 times in 15% sucrose/PBS and blocked in optimal cutting temperature (OCT) medium by snap freezing in isopentane cooled in liquid nitrogen.

IMMUNOCYTOCHEMISTRY

Immunocytochemistry using the alkaline phosphatase anti-alkaline phospatase (APAAP) technique was performed on cryostat sections of nasal biopsies as previously described.[12] Anti-human CD3, CD4, elastase and CD20 monoclonal antibodies (DAKO, Mississauga, Ontario, Canada) were used to phenotype T cells, neutrophils, and B cells respectively. Fast Red TR (Sigma-Aldrich, St. Louis, MO, USA) was the chromogen used to develop the reaction, therefore positive cells were identified as red under light microscopy. Negative control experiments were performed by replacing the primary Ab with an isotype-matched control.

IN SITU HYBRIDIZATION

IL-4, IL-5, IL-13, TNF-α and IFN-γ mRNA-positive cells were enumerated using *in situ* hybridization. For Figure 1, radioactive *in situ* hybridization with ^{35}S-labelled IL-4, IL-5, TNF, and IFN-γ cRNA probes was performed as previously described.[13] Briefly, sections were permeabilized with proteinase K (1 μg/ml) and Triton X-100. Prehybridization was performed in 50% formamide (Sigma) in 2 X SSC solution for 15 minutes at 37℃. Hybridization was performed overnight with ^{35}S-labelled antisense (complementary) and sense probes (identical to mRNA) in a humid chamber at 42℃. Post-hybridization washes of decreasing concentrations of SSC solution (4 X to 0.1 X SS) were conducted at 42℃. Non-specific binding was removed by washing in standard saline citrate, again at 42℃. Excess and unbound RNA probe was destroyed with RNase A (20 μg/ml). Slides were dipped in LM-2 emulsion (Amersham, Oakville, Canada), stored at 4℃ and exposed 11-15 days later. Autoradiographs were developed in Kodak D-19 solution (Eastman Kodak, Toronto, Canada) and counter-stained with Gill II Haematoxylin. Positive signals were identified as a collection of silver grains overly-

ing the cells (illustrated in Fig.2).

In separate experiments we looked at the effect of AIC or Amb-a-1 and ISS (unlinked) on Amb a 1-induced Th2-cytokine mRNA reactivity (Fig.3, *n* = 12). Here we used digoxigenin (Dig)-labelled *in situ* hybridization using digoxigenin-11-UTP labelled IL-4, IL-5, IL-13 and IFN-γ mRNA riboprobes as previously described.[14] Color development in Dig-labeled *in situ* hybridization was achieved by adding a freshly prepared substrate solution consisting of 0.175 mg X-phosphate-5-bromo-4-chloro-3-indoly phosphate (BCIP) and 0.37 mg nitroblue tetrazolium (NBT) salt per milliliter of equalization buffer to the slides (for 20-40 minutes at room temperature). Slides were transferred to TBS and washed in tap water, and counter-stained with hematoxylin for 5 seconds. Positive signals were identified by a purple stain on the cells under light microscopy. Negative control experiments using sense probes and RNase treatment before antisense probe applications were performed to confirm probe specificity.

QUANTIFICATION

Using an Olympus light microscope (Carson Group, Markam, Ontario, Canada) at X 200 magnification with an eyepiece graticule of 0.202 mm^2, slides were analyzed for positive signal in a blinded fashion by two independent examiners. Number of positive cells were counted and expressed as the mean number of cells *per* field.

STATISTICAL ANALYSIS

Inter-observer variance was examined by repeated measurements of (*n* = 3) of 5 samples. Intra-observer variance was tested by comparing the measurements of 10 different non-overlapping fields performed by two different observers. The intra-observer coefficient of variation was less than 5% for the counting in different samples measured 3 times. The intra-observer correlation co-efficient was more than 0.95 (*p* < 0.0001) for the counting of positive cells. The individual results were expressed as the mean number of positive cells per field ± SEM. The number of immuno-reactive cells within each group were compared using an ANOVA test, with a Dunnett's correction. Probability values of less than 0.05 were considered statistically significant.

RESULTS

Th1 AND Th2 CYTOKINE mRNA EXPRESSION

To investigate the effect of AIC on the local Th1- and Th2-mRNA cytokine reactivity in the nasal explant of ragweed-sensitized patients (*n* = 12), tissue was cultured with a) whole ragweed extract (RW), b) major ragweed allergen (Amb a 1), c) Amb a 1-linked-ISS (AIC), or d) tetanus toxoid (TT), a potent Th1-inducer (Fig. 1). In the control, unstimulated nasal explants, we detected constitutive IL-4, IL-5, TNF-α

A

B

C

D
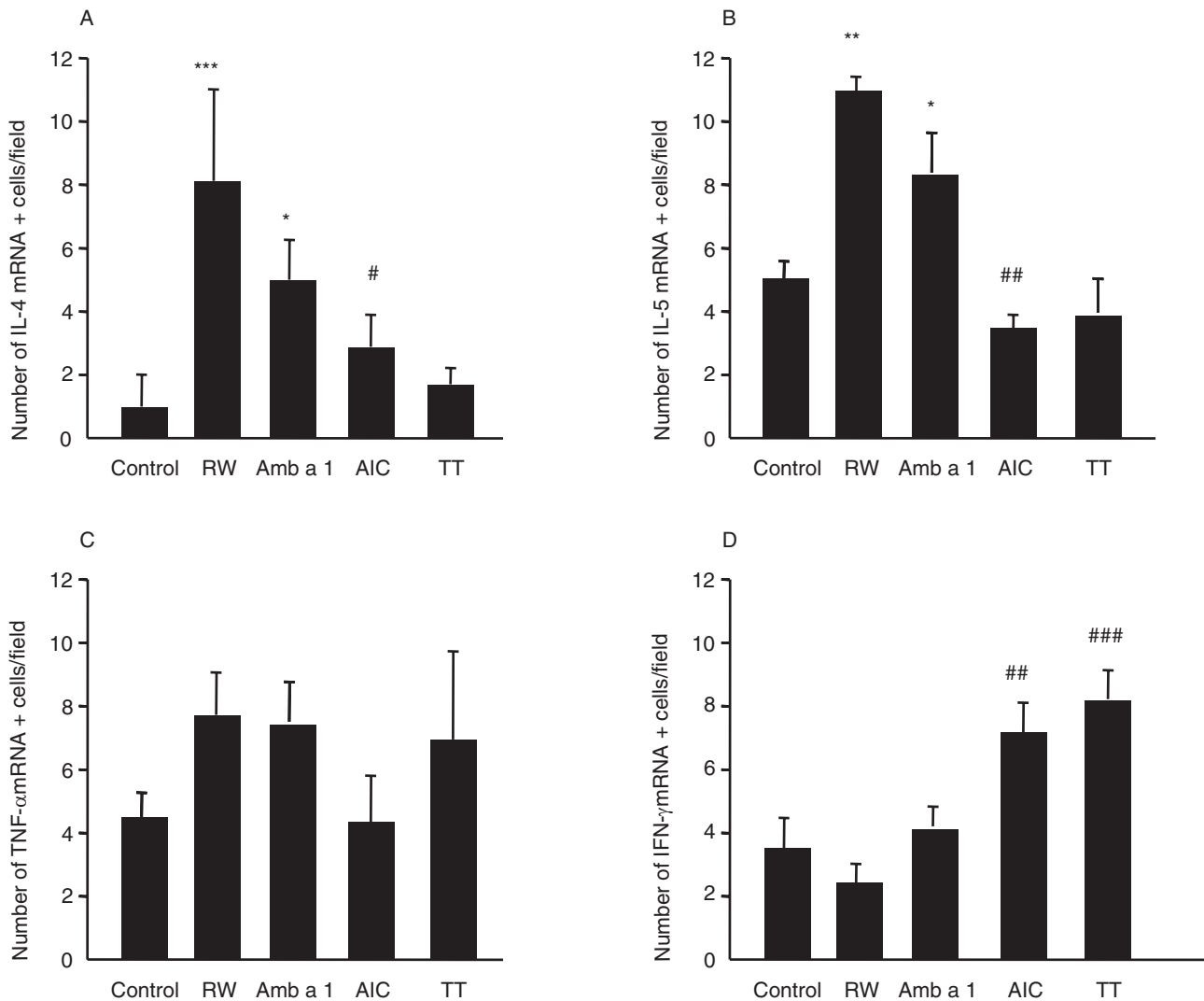
Fig. 1 The effect of AIC on allergen-induced cytokine mRNA expression. The number of IL-4 (**A**) , IL-5 (**B**) , TNF (**C**) and IFN-γ(D)mRNA positive cells in explanted nasal tissue (*n* = 12) following incubation in a) medium alone (control) , b) whole ragweed allergen (RW, 50 μg/ml), c) Amb a 1 (20 μg/ml), d) Amb a 1-linked-ISS (AIC, 5 μg/ml), or e) tetanus toxoid (TT, 0.5 Lf/ml) for 24 hrs. Cytokine mRNA immunoreactivity was assessed using radiolabeled ^{35}S riboprobes.*P < 0.05,**P < 0.01 and ***P < 0.001 versus control. #P < 0.05, ##P < 0.01 and ###P < 0.001 versus RW or Amb a 1 allergens.

and IFNγ mRNA+ cells in the tissue. Incubation with RW or Amb a 1 allergens significantly increased IL-4 (*P* < 0.001) (Fig. 1A) and IL-5 mRNA (*P* < 0.01) (Fig. 1B) expression with no change in IFN-γ mRNA reactivity (Fig. 1D). A trend for greater IL-4 and IL-5 mRNA expression was seen in explants stimulated with whole ragweed compared to Amb a 1 allergen however this did not reach significance (*P* = 0.062).

AIC failed to increase IL-4 or IL-5 mRNA however produced a significant increase in IFN-γ mRNA expression compared to control cultures (*P* < 0.01) and those stimulated with allergen (Amb a 1) (*P* < 0.01) or allergen extract (RW) (*P* < 0.01) (Fig. 1D). The increase in IFNγ mRNA was similar to that seen following stimulation with TT. TT produced no effect on IL-

4 or IL-5 mRNA expression (Fig. 1A, B). Although there was a trend for increases in TNF mRNA in tissue cultured with RW or Amb a 1 (*P* = 0.066) and decreases following incubation with AIC (*P* = 0.071), these changes were not statistically significant (Fig. 1C). Figure 2 are representative examples of *in situ* hybridization with ^{35}S-radiolabeled cRNA probes directed against IL-5 mRNA or IFNγ mRNA in nasal explanted mucosa stimulated with ragweed allergen (50 μg/ml) in the absence or presence of AIC.

In the second set of experiments (*n* = 12) we set out to determine whether AIC was more effective at modulating the local Th1- and Th2-immune response than both Amb a 1 and ISS added together (unlinked). Here we used digoxigenin-labeled ribo-
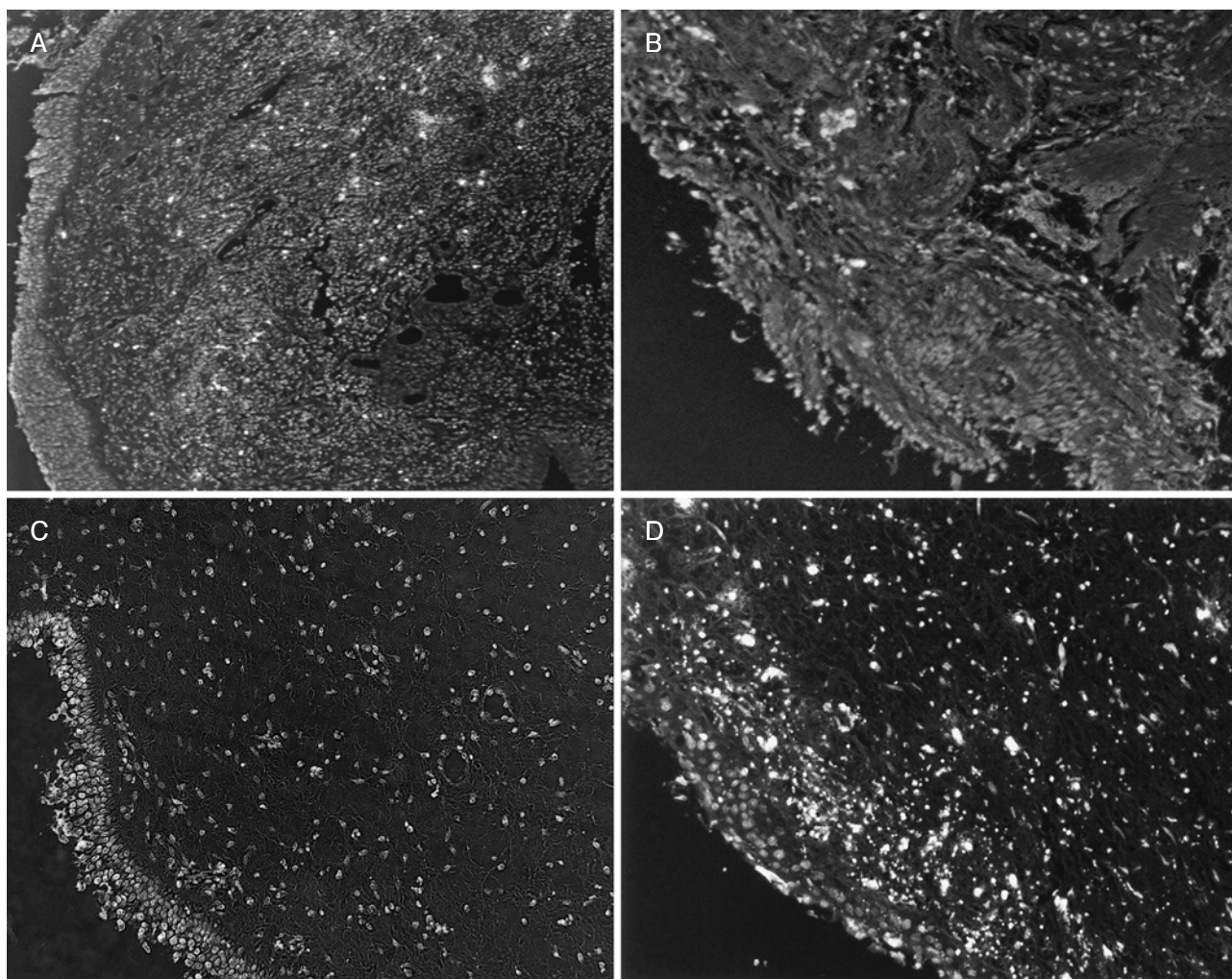
Fig. 2 AIC-induced inhibition of Th2- and increase in Th1-cytokine mRNA expression. Representative examples of *in situ* hybridization with ^{35}S-radiolabeled cRNA probes directed against IL-5 mRNA (top rows) or IFNγ mRNA (bottom rows) in nasal explanted mucosa stimulated with ragweed allergen (50 μg/ml) in the absence (**A** and **C**) or presence of AIC (5 μg/ml) (**B** and **D**). Positive mRNA signal was identified as silver grains over the cells. Magnification X 100.

probes to look at the local expression of IL-4, IL-5, IL-13, and IFN-γ mRNA positive cells (Fig. 3). In addition to IL-4, IL-5 and IFN-γ we also detected constitutive expression of IL-13 mRNA in the nasal tissue (Fig. 3C). These cells were located throughout the sub-mucosa. *Ex vivo* challenge with RW or Amb a 1 allergens induced significant increases in local IL-4, IL-5 and IL-13 mRNA but not when incubated with AIC. AIC induced a large increase in IFN-γ mRNA expression compared to control or allergen-stimulated cultures (Fig. 3D). Unlinked Amb a 1 and ISS produced similar results to AIC however both of its effects on Th1- and Th2- cytokine mRNA production were less effective (Fig. 3). The presence of exogenous Amb a 1 allergen in cultures had no effect on the ability of AIC to down-regulate local IL-4, IL-5 and IL-13 mRNA expression (Fig. 3A-C) or to up-regulate local IFN-γ mRNA (Fig. 3D). We found a strong posi-

tive correlation between IgG anti-Amb a 1 and IFNγ mRNA (r = 0.838, P = 0.002) as well as a strong negative correlation between IgG anti-Amb a 1 and IL-4 mRNA (r = -0.742, P = 0.001) or IL-5 mRNA (r = -0.788, P = 0.001) or IL-13 mRNA (r = -0.811, P = 0.002).

INFLAMMATORY CELL PROFILE
CD3+ pan T cells (48.2 ± 5 cells/field), CD4+ T cells (32.8 ± 2 cells/field), CD20+ B cells (25.5 ± 1 cells/field) and elastase+ neutrophils (25.3 ± 2 cells/field) were localized in unstimulated nasal explanted tissue. There was no significant difference in the local number of inflammatory cells following incubation with either allergens alone, AIC or unlinked Amb a 1 and ISS (data not shown).
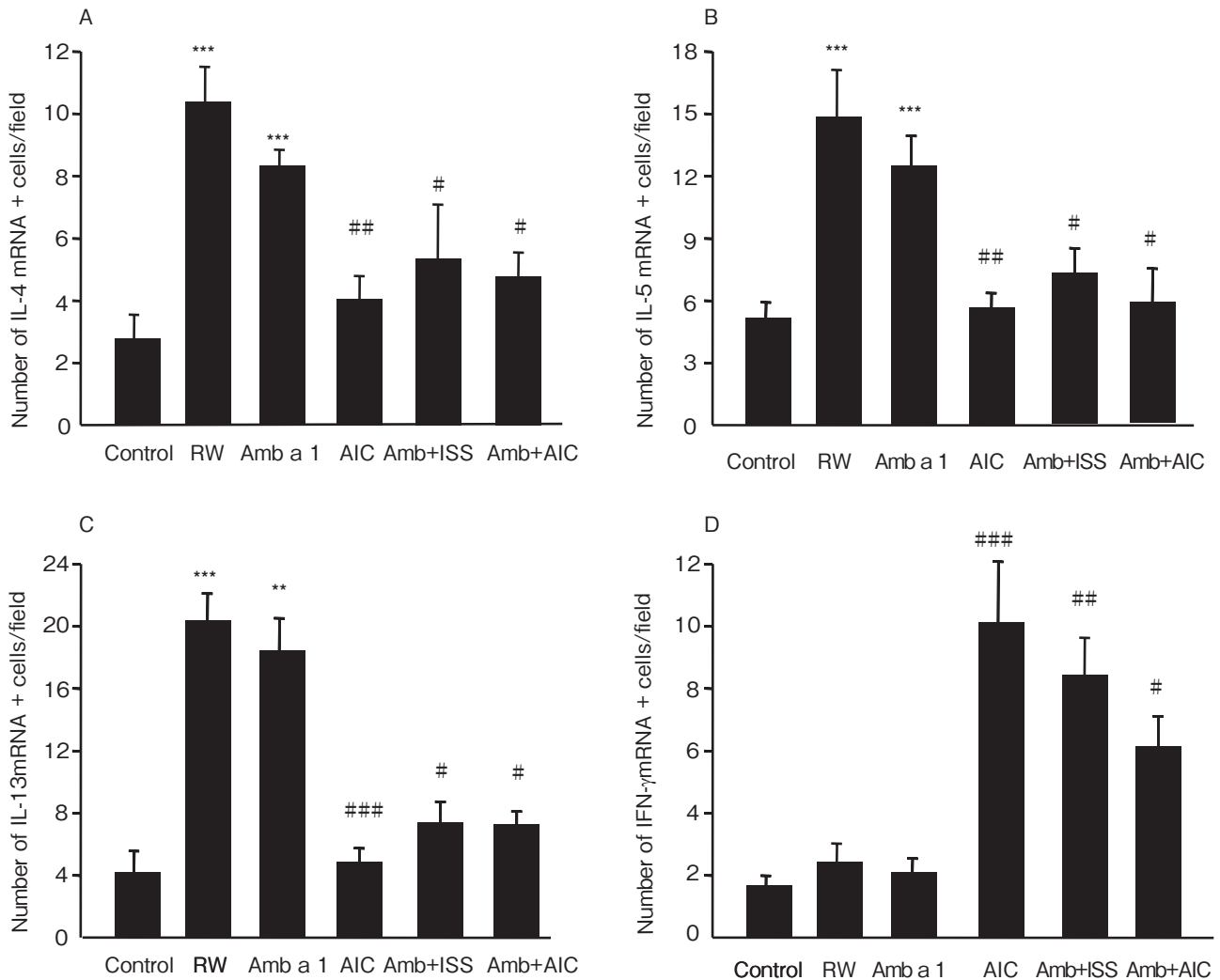
Fig. 3 The effect of allergen-linked-ISS versus addition of allergen with ISS in cultures on cytokine mRNA expression. The number of IL-4 (**A**), IL-5 (**B**), IL-13 (**C**) and IFN-γ(D)mRNA positive cells in explanted nasal tissue (*n* = 12) following incubation in a) medium alone (control), b) whole ragweed allergen (RW, 50μg/ml), c) Amb a 1 (20μg/ml), d) Amb a 1-linked-ISS (AIC, 5μg/ml), e) Amb a 1 + ISS (unlinked, 5μg/ml), or f) Amb a1+ AIC for 24 hrs. Cytokine mRNA immunoreactivity was assessed using digoxigenin labeled riboprobes. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus control. #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ versus RW or Amb a 1 allergens.

DISCUSSION

In this study we demonstrated that administration of Amb a 1-linked-ISS (AIC) inhibited the local allergen-driven Th2 immune responses and promoted Th1 cytokine expression in the explanted nasal mucosa of ragweed-sensitized patients. This was demonstrated by reduced expression of local tissue IL-4, IL-5 and IL-13 mRNA and increased expression of IFN-γ mRNA. A similar response was observed following culture with unlinked Amb a 1 and ISS, however this effect was weaker. These observations are consistent with our previous work showing short-course immunotherapy with AIC effectively promoted Th1- and down-regulated Th2-cytokine production in the nasal

biopsies of ragweed-sensitive patients following allergen challenge.[11] The lack of AIC effect on inflammatory cell number suggests its lack of effect on local cellular proliferation and is also consistent with our previous findings in the nasal mucosa following incubation with ragweed allergen.[9]

The proposed immunological mechanisms by which specific immunotherapy is effective include switching of allergen-induced Th2-immune responses towards Th1 response, induction of tolerance, induction of protective immunoglobulin responses (decrease of allergen-specific IgE and increases in IgG antibodies, mainly IgG4) and induction of regulatory cells (reviewed in[15]). In our forerunner separate clinical study, we have shown AIC immunotherapy to ef-

fectively increase protective IgG anti-Amb-a-1 antibody responses in ragweed-sensitive patients and to effectively drive the immune responses away from Th2 phenotype.[11] In this study we provide novel evidence for AIC directly affecting the local Th1/Th2 cytokine milieu in the nasal tissue without relying on systemic recruitment of inflammatory cells to the site of inflammation. Recent evidence from our group suggests that these responses may be, at least in part, modulated by the resident nasal Treg cells.[16] In that study we used the same patients as described here and have shown AIC immunotherapy to significantly increase the number of infiltrating CD4+CD25+ cells into the nasal mucosa of allergic patients post-seasonal ragweed-pollen exposure.[16] Furthermore, in that study a strong trend towards increased IL-10 immunoreactivity was observed, the source of which is likely to be CD4+CD25+ Treg cells. Although we did not perform IFNγ +CD4+CD25+ triple immunostaining, T cells are also the most likely source of AIC-induced IFNγ mRNA production as IFNγ staining was localized predominantly to small mononuclear cells. Furthermore, we know that CpG-induced activation of TLR9 stimulates IFN-γ production from T cells *via* indirect activation of pDC production of IL-12 and IFN-α.[17] It is interesting to note that following cell stimulation with CpG DNA, TLR9 and the DNA are found in the same endocytic vesicles,[18] suggesting that CpG modification of AIC may directly facilitate increased uptake of this antigen into the cell. This would allow activation of the same antigen presenting cells to present Amb a 1 to T cells in a Th1 biasing context induced by the CpG motifs.

Co-culture of Amb a 1 with ISS-ODN also induced a Th1 response in nasal tissue, however it was weaker than that induced with the corresponding ISS-conjugate. Tighe and colleagues[6] have similarly shown that co-injection of Amb a 1 with ISS-ODN in mice induced a weak Th1 response, albeit much lower than that seen with AIC conjugate. This was demonstrated by a lower IgG2α titer, and a significantly lower level of IFN-γ secreted by T cells in mice co-injected with a mixture of Amb a 1 and ISS.[6] Administration of Amb a 1 with ISS may result in the *in vivo* dissociation of the ISS from the antigen, resulting in reduced efficiency. Chemical conjugation of ISS to allergen on the other hand, confers allergen properties that make it an optimal candidate for immunotherapy such as the induction of a rapid and significant allergen-specific IFN-γ release as well as the IgG antibody formation with injections of low doses of allergen. These findings indicate that conjugation of ISS to antigens may be necessary for successful induction of immune responses to weak or very low doses of antigens. The reason for this may be that ISS and allergen need to be delivered to the same antigen-presenting cell inducing a Th1-biasing microenvironment as mentioned above.[6] The reduction

in CpG dose will also reduce the risk of potential adverse effects of CpG administration, such as theoretical risks of induction of auto-immune disease or inflammatory responses.[6,17,19]

The results of this study demonstrate the effectiveness of allergen-linked immunostimulatory DNA in directly stimulating a local, Th1 response and decreasing an allergen-specific Th2 response, without any significant effect on local cellular inflammation. Although we have previously described the effectiveness and safety of AIC immunotherapy in these same patients,[11] here we demonstrate for the first time that AIC is capable of modifying local immune responses in the nose and therefore without any cellular recruitment to this site. Together, these data support a role for direct mucosal application of AIC as a possible new form of available immunotherapy for ragweed-sensitized patients.

ACKNOWLEDGEMENTS

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