Modulation of immunogenicity and allergenicity by controlling the number of immunostimulatory oligonucleotides linked to Amb a 1

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Background: Immunostimulatory DNA sequences (ISS) are potent immunomodulators that can drive T_H1 responses to antigens or allergens. This effect can be dramatically enhanced by direct linkage of ISS to the protein.
Objective: Evaluate the effects of the number of ISS bound to the major ragweed allergen Amb a 1 on immunogenicity and allergenicity.
Methods: Immunogenicity in mice and allergenicity using PBMC or sera from subjects with ragweed allergy were assayed.
Results: Both antibody induction *in vivo* and antibody recognition *in vitro* were highly sensitive to the number of ISSs linked. IgE recognition of Amb a 1 in competitive ELISA or histamine release assays was inhibited by ISS linkage and showed an inverse relationship to the number of ISSs bound. Type and magnitude of antibody induction in mice was also highly dependent on the number of ISS bound. At the highest ISS to protein ratios, antibody induction was very low. Moderate ISS to protein ratios induced high antibody responses in which IgG_{2a} generally predominated. Low ISS to protein ratios produced the highest overall antibody responses in which IgG_1 predominated. In contrast, varied ISS to protein ratios did not affect T-cell responses. In both *in vivo* mouse studies and *in vitro* human PBMC studies, all ISS to protein ratios evaluated induced similar responses represented by high levels of IFN-γ and low levels of T_H2 cytokines.
Conclusion: Controlling the number of ISS bound to a protein allows manipulation of antibody recognition and induction while retaining the potent T_H1 properties of an ISS-linked protein.
Clinical implications: Immunostimulatory DNA sequence–linked Amb a 1 conjugate represents a safe, novel therapeutic approach for treating ragweed allergy. (J Allergy Clin Immunol 2006;118:504-10.)

Key words: *Allergy, immunostimulatory oligonucleotide, linked, immunogenicity, ragweed, Amb a 1, allergy vaccine*

Immunostimulatory DNA sequences (ISS) containing CpG motifs are recognized by Toll-like receptor 9 and exhibit a variety of immunostimulatory and immunomodulatory properties (reviewed by Klinman et al[1]). ISS stimulate the production of T_H1-type cytokines such as IL-12 and IFNs from a variety of cells such as dendritic cells, macrophages, and natural killer cells. ISS also stimulate B-cell proliferation and immunoglobulin secretion as well as activation of antigen presenting cells. ISS have been demonstrated to have potent T_H1 adjuvant properties when used for immunization associated with either DNA or protein vaccines. The ability to redirect allergic responses in several mouse allergy models has been demonstrated by using ISS.[2-5]

Several studies have demonstrated that direct linkage of ISSs to antigens or allergens can dramatically enhance the effect of ISSs in modulating the immune response to the linked proteins. Conjugation of ISS to Amb a 1, the major allergen of short ragweed pollen, effectively redirects the immune response from a T_H2-type response normally induced by the antigen alone and promotes a strong T_H1 response.[6-8] In PBMCs from people with ragweed allergy, this is illustrated by a shift in cytokine response from high IL-4, IL-5, low IFN-γ, to low IL-4, IL-5, high IFN-γ.[6] Related studies with protein-ISS conjugates of β-galactosidase or HIV gp120 have induced much higher levels of IFN-γ, cytotoxic T lymphocyte activity, and IgG_{2a} antibody than antigens alone, antigens mixed with ISS DNA, or DNA vaccines.[9] Ovalbumin (OVA) linked to ISS was shown to generate strong, T_H-independent cytotoxic T lymphocyte responses that provided protection against ovalbumin-expressing tumor cells.[10] OVA-ISS conjugate was also shown to be 100-fold more efficient than OVA mixed with ISS in inducing T_H1 responses and inhibiting airway eosinophilia and hyperresponsiveness in a mouse ovalbumin asthma model.[11]

Although initial studies have demonstrated that direct linkage of ISS to antigens or allergens can dramatically

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Abbreviations used
AIC: Amb a 1 linked ISS conjugate
AIC-H: AIC with high ISS-to-protein ratio
AIC-L: AIC with low ISS-to-protein ratio
AIC-M: AIC with moderate ISS-to-protein ratio
ISS: Immunostimulatory DNA sequence(s)
MW: Molecular weight
OVA: Ovalbumin
SAv-HRP: Streptavidin-horseradish peroxidase conjugate
TMB: Tetramethyl benzidine

enhance the influence of ISS on immune responses to the linked protein, little information is available on how specific protein-ISS linkages can be optimized to achieve desired immune responses. In the current study, we investigated how the number of ISS linked to the Amb a 1 protein affects the Amb a 1–specific immunogenicity of mice *in vivo*, and PBMCs from subjects with ragweed allergy *in vitro*.

METHODS

Preparation and characterization of Amb a 1–ISS conjugates

Amb a 1 was purified from short ragweed pollen, *Ambrosia artemisiifolia*, obtained from Greer Laboratories (Lenoir, NC) using a proprietary process that includes standard extraction and chromatographic techniques. The resulting protein is more than 90% pure as determined by reverse-phase HPLC.

Oligonucleotides containing either an immunostimulatory CpG sequence (1018 ISS: TGACTGTGAACGTTCGAGATGA) or control oligonucleotides without CpGs (1019–non-ISS: TGACTGTGAAGGTTAGAGATGA, 1040–non-ISS: TGACTGTGAACCTTAGAGATGA) were synthesized by standard phosphoramidite chemistry with a chemically protected reactive group at the 5′ end of the molecule by Avecia Biotechnology (Milford, Mass) or Boston Biosystems (Bedford, Mass). The 1018 ISS was also synthesized without the 5′ reactive group for use as an unlinked reagent.

For conjugation, Amb a 1 was activated by using a heterobifunctional chemical linker, and excess linker removed. The reactive group on the 1018 ISS was deprotected and the protecting group was also removed. The 2 activated biomolecules were then mixed in various ratios to generate conjugates with low, moderate, and high molar ratios of oligonucleotide per Amb a 1. Excess oligonucleotide was removed from the Amb a 1-1018 ISS conjugate (AIC). Control Amb a 1–non-ISS conjugates were also made by this method using mixes of the activated protein and oligonucleotide materials to generate linked materials with moderate ratios of oligonucleotide per Amb a 1.

The AIC size distribution relative to protein standards of known molecular weight was determined by SDS-PAGE and densitometry. The protein component of the sample was visualized by Coomassie staining whereas the oligonucleotide component was detected by DNA-specific silver stain. The 1018 ISS to Amb a 1 molar ratio, which characterizes the average distribution of AIC species, is determined by spectrophotometric measurement. 1018 ISS content is determined by using UV absorption at 260 nm. Amb a 1 protein content is measured against a standard curve with the bicinchoninic acid assay.

Mice: Immunization, antibody and cytokine measurements

Female BALB/c mice (H-2^d, 8-12 weeks) were purchased from either Jackson Laboratory (Bar Harbor, Me) or Charles River Laboratory (Wilmington, Mass). Animal studies were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited animal facility and were approved by their animal care and use committee according to the "Guide for the Care and Use of Laboratory Animals." Mice were injected twice intradermally in the tail at a 2-week interval. Amb a 1 and corresponding conjugates were diluted in PBS.

Animals were immunized with 45 individual preparations of AIC having ISS to protein ratios ranging from 2.0 to 6.4 to investigate the relative immunogenicity of Amb a 1 linked with variable amounts of 1018 ISS. Although the protein dose was held constant at 1 μg of Amb a 1, the dose of ISS differed according to the degree of conjugation. Therefore, mice received 0.4 to 1.3 μg 1018 ISS depending on the ISS to protein ratio of the individual AIC preparation. Control animals were immunized with 10-μg doses of unconjugated Amb a 1, because the unmodified form of the allergen is poorly immunogenic in mice. An additional control group was immunized with 1-μg doses of Amb a 1 mixed with but not conjugated to 1 μg 1018 ISS. Ten mice per group were immunized, and all lots were tested at least once, most lots were tested 2 or more times.

Amb a 1–specific IgG_1 and IgG_{2a} titers were measured 2 weeks after the second immunization using ELISA. Briefly, serial dilutions of mouse serum samples were analyzed by ELISA in 96-well round-bottom plates coated with Amb a 1. Goat antimouse IgG_1 or IgG_{2a} biotin-conjugated antibody was used as the secondary antibody. Streptavidin-horseradish peroxidase conjugate (SAv-HRP) was used for detection. The assay was developed with tetramethyl benzidine (TMB), and the absorbance values were determined at 450 nm with background subtraction at 650 nm on a microplate reader. The titer was defined as the reciprocal of the serum dilution that gave an ELISA absorbance of 0.5 OD using 4-parameter analysis. All samples were tested in duplicate wells on separate plates, and the titers were reported as the mean of the 2 values.

Cytokine production was measured 4 weeks after the second immunization. Splenocytes from individual mice, 5×10^5 cells/well, were stimulated *in vitro* with Amb a 1 at 25 μg/mL. After a 4-day stimulation, culture supernatants were harvested and tested for IL-5 and IFN-γ production by capture ELISA using an anticytokine mAb. Biotin-labeled anticytokine mAbs were used as secondary antibodies. SAv-HRP was used for detection, and the assay was developed with TMB. Concentration was calculated from a standard curve assayed on each plate. The absorbance values were determined at 450 nm with background subtraction at 650 nm on a microplate reader. All samples were tested in duplicate wells on separate plates, and the concentrations reported as the mean of the 2 values.

Cytokine induction in human PBMCs

The procedure was described in a previous publication.[6] Briefly, PBMCs from individuals with ragweed allergy were cultured at 2×10^6/mL for 6 days with 5 μg/mL Amb a 1 or Amb a 1 linked with ISS at ISS to protein ratios of 2.8 (AIC-L), 4.1 (AIC-M), or 5.6 (AIC-H). Cultures were harvested on day 6, and supernatants were removed and stored frozen until assayed for IFN-γ content by ELISA. Some of the cells were washed, counted, and restimulated at 2×10^6/mL per well in 24-well plates for 24 hours with 5 μg/mL phytohemagglutinin and 50 ng/mL phorbol myristate acetate. Supernatants were then removed and stored frozen until assayed for IL-4, IL-5, and IL-13 using Cytoscreen ELISA kits from Biosource (Camarillo, Calif).

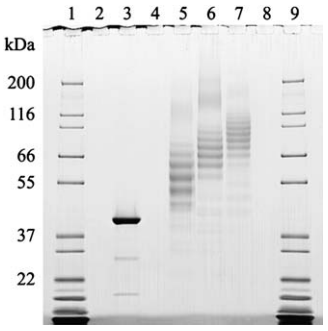

FIG 1. The linkage of 1018 ISS to Amb a 1 results in populations of AIC species representing a low, moderate, and high 1018 ISS to Amb a 1 ratio. Sample load on SDS-PAGE as follows: *lanes 1 and 9,* Mark12 Wide Range Protein Standard (Invitrogen, Carlsbad, Calif); *lanes 2, 4, and 8,* blank; *lane 3,* Amb a 1; *lane 5,* AIC-L; *lane 6,* AIC-M; *lane 7,* AIC-H.

IgE binding competition

Amb a 1–specific IgE titers of sera from donors with ragweed allergy was determined with an antihuman IgE secondary antibody using the isotype methodology described. Serial dilutions of Amb a 1 or ISS-linked Amb a 1 were incubated with this human serum in polypropylene 96-well plates for 1 hour at room temperature to allow prebinding to IgE. These prebound samples are then transferred to Amb a 1–coated ELISA plates and incubated 30 minutes at room temperature to allow remaining available IgE to bind. Plates were washed and detected with polyclonal, biotinylated antihuman IgE. After incubation with SAv-HRP and development with TMB substrate, the ODs of each well were measured. The concentration of the pretreatment required to reduce the ELISA signal by 50% was calculated and is inversely related to the ability to bind IgE.

Histamine release assays

Venous blood collected from donors with ragweed allergy (n = 4) was mixed with a solution of dextrose, EDTA, and dextran to sediment erythrocytes as previously described.[12] The leukocyte supernatant was used as a source of basophils. Cells were mixed with varying concentrations of Amb a 1 or ISS-linked Amb a 1 and incubated for 45 minutes at 37°C with mixing every 15 minutes. The cells were pelleted by centrifugation, and the histamine content of the supernatant was determined with an RFA 300 autoanalyzer from Astoria-Pacific International (Clackamas, Ore). Control tubes for determining the background histamine release contained cells and buffer only. Background histamine release was usually not more than 2% of the total cellular histamine level, which was determined by lysis of untreated cells with 2% $HClO_4$.

RESULTS

Physicochemical characterization of Amb a 1 linked to ISS at different ISS to protein ratios

Amb a 1 contains as many as 19 potential sites capable of accepting linkage to an ISS oligonucleotide. By controlling the ISS to protein ratio used during the conjugation reaction, it is possible to generate Amb a 1 linked to a defined range of 1018 ISS oligonucleotides. The number of ISS linked to Amb a 1 can be calculated on the basis of mass of the antigen using SDS-PAGE.

Purified Amb a 1 migrates with an apparent molecular weight (MW) of 41 kd (Fig 1, *lane 3*); the 2 lower MW species visible on the gel correspond to specific proteolytic fragments. Amb a 1 conjugated at low ISS to protein ratios migrate at MWs ranging from 47 to 68 kd *(lane 5)*. On the basis of the mass difference with purified Amb a 1, AIC with low ISS-to-protein ratio (AIC-L) consists of Amb a 1 linked to 1-5 1018 ISS with an average ISS to Amb a 1 ratio of 2:1. The major species in AIC with moderate ISS-to-protein ratio (AIC-M), conjugated at moderate ratios, range from 58 to 91 kd, or 3-8 1018 ISSs linked to Amb a 1 *(lane 6)*, with the average ISS to Amb a 1 ratio of 4.6:1. Finally, the major species in AIC with high ISS-to-protein ratio (AIC-H) range from 67 to 104 kd, corresponding to 5 to 10 linked 1018 ISSs and an average ISS to Amb a 1 ratio of 5.9:1 *(lane 7)*. AIC-H, M, and L exhibited a similar distribution of Amb a 1-1018 ISS conjugates visualized on SDS-PAGE as a population generally consisting of 5 major species (Fig 1, *lanes 5-7*).

Antibody responses induced by AIC are dependent on the degree of conjugation

In this work, AIC lots have been grouped as follows for reporting and discussion purposes. AIC lots having an average ISS to protein ratio ranging from 2.0 to 3.0 are referred to as AIC-L (low), AIC lots having an average ISS to protein ratio ranging from 3.8 to 4.7 are referred to as AIC-M (moderate), and AIC lots having an average ISS to protein ratio ranging from 5.3 to 6.4 are referred to as AIC-H (high). The gap among the AIC-H, AIC-M, and AIC-L oligonucleotide to protein ratios is a result of no conjugates having been made in those ranges. There were 4 lots of AIC-L, 28 lots of AIC-M, and 13 lots of AIC-H tested. The 13 AIC-H lots were tested in a total of 330 mice, the 28 AIC-M lots in 980 mice, and the 4 AIC-L lots in 100 mice spread over several studies.

The mean antibody responses for all groups of mice tested with AIC-H, AIC-M, and AIC-L are shown in Fig 2, *A*. Immunization with Amb a 1 alone induced predominantly an IgG_1 antibody response. Immunization with Amb a 1 mixed with ISS resulted in a very low antibody response that was predominantly IgG_1. Animals immunized with AIC-H induced very low antibody titers overall. The IgG_1 titers induced with AIC-H were significantly lower than titers induced by the Amb a 1 + ISS mix ($P < .001$), whereas the IgG_{2a} titers did not differ significantly. In contrast, immunization with AIC-M produced a significantly enhanced IgG_{2a} response ($P < .001$) compared with mix. Immunization with AIC-L induced the highest overall antibody responses, significantly enhancing both IgG_{2a} and IgG_1 titers by more than 73-fold and 15-fold respectively, compared with titers induced by the Amb a 1 + ISS mix. Amb a 1–specific IgE responses did not reach detectable levels in any AIC immunized group using this immunization protocol (data not shown).

The degree of ISS substitution onto Amb a 1 dramatically affected both the magnitude and the quality of the antibody responses induced to the ragweed allergen. The

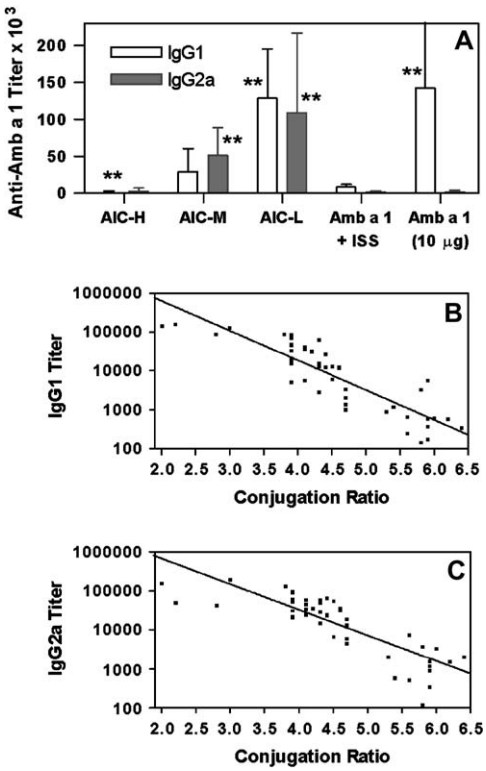

FIG 2. Amb a 1–specific antibody responses in immunized mice are inversely correlated with the number of linked ISS. **A,** Averaged titers ± SDs from all AIC-H, M, and L lots tested (n = 45 lots with ≥10 mice/lot); $^{**}P < .001$ compared with Amb a 1 + ISS. **B** and **C,** Mean Amb a 1–specific IgG_1 and IgG_{2a} titers plotted against the ISS to protein ratio for each individual AIC lot tested.

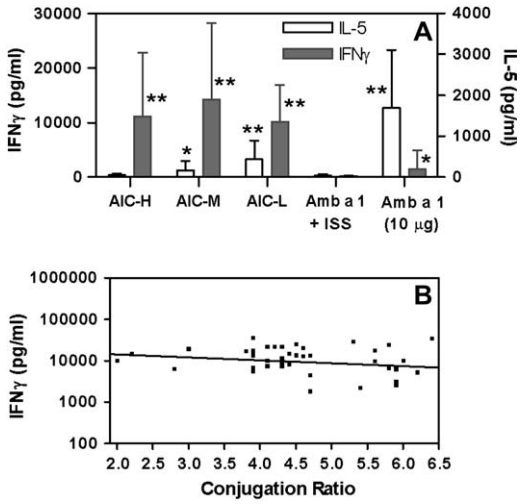

FIG 3. AIC induces Amb a 1–specific IFN-γ response in immunized mice regardless of the ISS to protein ratio. **A,** Averaged IFN-γ responses ± SDs from all AIC-H, M, and L lots tested (n = 45 lots with ≥10 mice/lot); $^{*}P < .05$, $^{**}P < .001$ compared with Amb a 1 + ISS. **B,** Mean Amb a 1–specific IFN-γ responses plotted against the ISS to protein ratio for each individual AIC lot tested.

Amb a 1–specific IgG_1 and IgG_{2a} responses versus the ISS to Amb a 1 ratio for each AIC lot tested are shown in Fig 2, *B and C*. When analyzed as a function of the oligonucleotide to protein ratio, there is a strong inverse correlation between antibody response and the degree of conjugation. At conjugation ratios of 2:1, antibody titers were greater than 10^5, whereas at conjugation ratios of 6:1, titers were at least 1000-fold lower. In general, beyond a 2:1 ratio of ISS to Amb a 1, there was a 5-fold decrease in the magnitude of the IgG_1 and IgG_{2a} titers with the linkage of each additional 1018 ISS oligonucleotide.

Immune responses of mice to 1 μg Amb a 1 linked with non-ISS control oligonucleotides containing no CpG sequences were also evaluated. The oligonucleotide to protein ratio was similar for the ISS and non-ISS conjugates used in the study. Antibody responses for the AIC-M and Amb a 1 immunized mice were similar to those previously seen, but the non-ISS conjugates did not induce an Amb a 1–specific IgG_{2a} response, nor was the IgG_1 response enhanced (data not shown).

IFN-γ responses induced by AIC in mice and human beings are independent of the degree of conjugation

BALB/c mice immunized with Amb a 1 exhibit a prototypical T_H2-type T-cell response characterized by secretion of high levels of IL-5 and low levels of IFN-γ (Fig 3, *A*). Immunization with AIC-H, M, or L induced characteristic T_H1 responses evidenced by significant IFN-γ production and low levels of IL-5. In contrast with the antibody responses, IFN-γ responses were not dependent on the degree of 1018 ISS conjugation (Fig 3, *B*); however, the IL-5 responses were inhibited in an ISS dose-dependent fashion (Fig 3, *A*). Although IL-5 responses to Amb a 1 can be suppressed simply by mixing the antigen with 1018 ISS, enhancement of IFN-γ responses required linkage of 1018 ISS to the antigen.

Mice immunized with Amb a 1 non-ISS control conjugates induced IL-5 responses similar to animals immunized with Amb a 1 alone, and only low-level induction of IFN-γ, indicating that the effects of linked oligonucleotide are sequence-specific (data not shown). ISS sequences containing CpG motifs are required to induce high IgG_{2a} and IFN-γ responses, and to reduce IL-5 responses in mice.

The effect of the degree of ISS conjugation on cellular immune responses was further investigated using PBMC from subjects with ragweed allergy. T-cell responses were determined by measuring the IL-4, IL-5, IL-13, and IFN-γ secreted by PBMCs cultured for 6 days in the presence of Amb a 1, AIC-H, M, or L. As expected, incubation with Amb a 1 induced T_H2-skewed cytokine responses with IL-5 and IL-13 secretion averaging nearly 3500 and 900 pg/mL, respectively, IL-4 secretion in excess of 100 pg/mL, and IFN-γ responses less than 20 pg/mL (Fig 4). In contrast, PBMCs stimulated with all forms of AIC exhibited a phenotypic T_H1 cytokine response. AIC-H, M, and L all induced similar responses; secretion of IL-5 and IL-13 was reduced approximately 7-fold compared with Amb a 1–stimulated cells, and IL-4 secretion was near

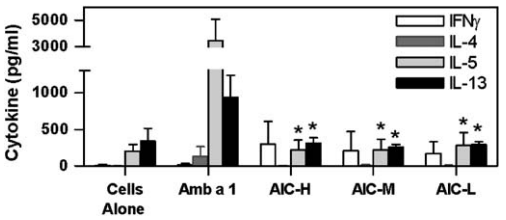

FIG 4. Amb a 1 linked to ISS at different ratios provide similar T_H2 to T_H1 shift in PBMC from people with ragweed allergy (n = 3); $*P < .05$ compared with Amb a 1.

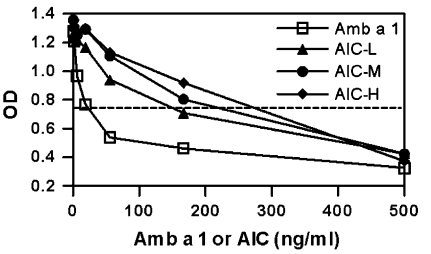

FIG 5. Increasing the amount of linked ISS reduces the ability of Amb a 1 to compete for binding of IgE from a human subject with allergy. AIC-L, M, H, or Amb a 1 was allowed to prebind to IgE sera from a subject with ragweed allergy before being added to an Amb a 1–coated ELISA plate. OD signal is inversely proportional to the ability of the pretreatment material to bind to IgE.

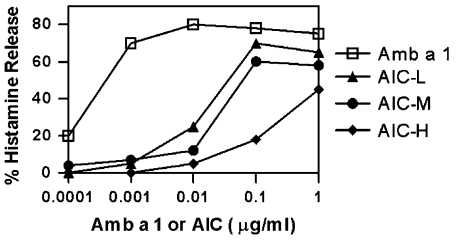

FIG 6. Increasing the amount of linked ISS reduces the ability of Amb a 1 to induce histamine release from leukocytes of subjects with ragweed allergy. Histamine release for a representative subject after *in vitro* cell treatment with Amb a 1 or AIC-L, M, or H.

the limit of detection. More importantly, IFN-γ production was increased 10-fold. Thus, as seen with *in vivo* cytokine induction in the mouse, human T-cell responses *in vitro* to ISS-linked Amb a 1 are not sensitive to the degree of ISS conjugation and respond with virtually the same T_H1-type response across a wide range of conjugation ratios.

Inhibition of human IgE binding to Amb a 1 is directly related to the number of ISS oligonucleotides conjugated to the allergen

The effect of ISS conjugation ratios on Amb a 1 binding of IgE, a key event associated with triggering an allergic response, was determined by using a competition ELISA. In this assay, unmodified Amb a 1 effectively competed for IgE binding reducing the ELISA signal by 50% at a concentration of 24 ng/mL (Fig 5). The capacity of AIC-L to bind IgE was considerably reduced requiring concentrations of 158 ng/mL to achieve 50% signal reduction. Increasing the degree of ISS conjugation further reduced IgE binding. For AIC-M, 218 ng/mL, and for AIC-H, 277 ng/mL was required to achieve 50% signal reduction. Relative to unmodified Amb a 1, AIC-L, M, and H required 6.6, 9.1, and 11.5-fold more protein to achieve 50% signal inhibition.

Modulation of histamine release by basophils from subjects with ragweed allergy is dependent on the amount of ISS conjugated to Amb a 1

It has previously been demonstrated that linking ISS to Amb a 1 reduces the ability of Amb a 1 to induce histamine release from basophils of donors with ragweed allergy.[8] In the current study, assays to measure the levels of histamine release from basophils of donors with ragweed allergy were performed to assess directly the effect of different ISS to Amb a 1 ratios on modulating protein allergenicity. The results for all dilutions on a representative leukocyte sample are shown in Fig 6. The leukocytes were very sensitive to unmodified Amb a 1, with high levels of histamine being released at very low concentrations of Amb a 1. In 4 different donors, the amount of Amb a 1 required to stimulate a 40% release of histamine averaged 0.00025 μg/mL (Table I). Linking ISS to Amb a 1 reduced the sensitivity of the cells to the modified allergen by 78-fold, 156-fold, and >1667-fold for the AIC-L, M, and H forms, respectively. Histamine release from basophils of donors with ragweed allergy is an IgE-mediated activity. These results imply that linking ISS to allergen reduces the ability of IgE to bind to or cross-link the allergen and that this effect is inversely related to the amount of ISS linked to the allergen.

DISCUSSION

The results from this study confirm earlier reports that direct linkage of ISS to antigens or allergens dramatically enhances the ability of ISS to modify antibody and T-cell responses to the proteins.[6,8-11] In the current work, these studies were extended to determine how the number of ISS oligonucleotides linked to a protein affects its antigenic and immunogenic properties. The results show that the number of oligonucleotides linked to the protein has dramatic effects on antibody induction and recognition but very little effect on the T-cell responses. Shirota et al[13] and our own work (data not shown) have demonstrated increased uptake of ISS-linked antigen by dendritic cells. Linked ISS may serve to enhance cell uptake of AIC nonspecifically as a result of increased size or charge, because we have seen the same effect with non–ISS-linked antigens. However, our studies with non–ISS-linked proteins have shown that these conjugates do not modulate antibody or cytokine responses, and therefore, the effect is ISS-specific.

Linking ISS to Amb a 1 reduces the ability of IgE antibody to bind the protein and correspondingly increases

TABLE I. Concentration of AIC-L, M, and H required to induce 40% histamine release from human leukocytes compared with Amb a 1

Subject	Amb a 1 (μg/mL)	AIC-L (μg/mL)	Fold increase	AIC-M (μg/mL)	Fold increase	AIC-H (μg/mL)	Fold increase
1	0.0002	0.020	100	0.030	150	0.500	2500
2	0.0003	0.010	33	0.030	100	>0.10	>333
3	0.0002	0.018	90	0.028	140	>0.10	>500
4	0.0003	0.026	87	0.070	233	>1.00	>3333
Mean	—	—	78	—	156	—	>1667

the amount of allergen required to induce histamine release from basophils of donors with ragweed allergy. There is an inverse relationship between the number of ISS oligonucleotides that are linked and both levels of IgE binding and the sensitivity of histamine release. The reduced recognition by IgE of Amb a 1–ISS conjugates appears to be the result of steric hindrance, chemical blocking, or alteration of B-cell epitopes rather than any specific activity of the ISS, because linkage of non-ISS oligonucleotide to Amb a 1 was equally effective in reducing the allergenicity of the antigen.[8] Because the linked oligonucleotide would most likely be presented on the surface of the protein, it is likely that the oligonucleotides mask the epitopes recognized by the antibody. This idea is supported by the finding that free ISS had no effect on histamine release induced by Amb a 1.[8]

The consequences of ISS conjugation on blocking B-cell epitopes were also evident in the murine immunogenicity studies. Linkage of high amounts of ISS to Amb a 1 nearly abolished the antibody response, whereas decreasing the ISS to protein ratio resulted in enhanced humoral responses consisting of Amb a 1–specific IgG_1 and IgG_{2a} antibodies. These results suggest that ISS linkage represents a tradeoff to the immune system. Although ISS conjugation at low to moderate ratios causes some blocking of B-cell epitopes, the potent immunostimulatory affects of the linked oligonucleotides overcomes this. Although non–ISS-linked Amb a 1 would not be allergenic, in the absence of immunostimulatory activity the antigen would not induce T_H1 responses and therefore would not be expected to desensitize the immune system to subsequent re-exposure to Amb a 1.

The effect of linking different numbers of ISS oligonucleotides to Amb a 1 on T-cell response appears to be very different from the effect on antibody-mediated events. Linking ISS to proteins dramatically increases T_H1 and reduces T_H2 responses as measured by IFN-γ and IL-5 induction in both animals and human PBMCs. The ability of ISS linked Amb a 1 to induce antigen-specific IFN-γ responses does not appear to vary significantly using AIC with ISS to protein ratios ranging from 2.0 to 6.4. Although there was evidence of a dose-dependent suppression of IL-5 production, the changes were not as dramatic and did not reach statistical significance. For Amb a 1, induction of IFN-γ T-cell responses did require ISS linkage to the antigen because Amb a 1 mixes with ISS doses as high as 100 μg were not able to induce IFN-γ. This effect may be antigen-specific, however, because we have seen with other proteins that IFN-γ could be induced with high ISS mix doses (data not shown).

The effect of differential ISS to protein ratios in this study were all measured by using a single protein species (Amb a 1). Horner et al[14] have found similar results in a mouse anaphylaxis model in which increasing the number of ISS oligonucleotides bound to ovalbumin reduced the anaphylactic properties of the protein while retaining potent T_H1 immunogenicity. Using ovalbumin conjugates with high (5.5) and low (2.5) ISS to protein ratios, these studies noted a slight reduction in IFN-γ responses with the high conjugate. Our current work using 13 separate AIC-H preparations shows some variation in response levels, but the variation is not significant. Although this effect may vary for different proteins, the data from this study imply that ISS linkage does not deleteriously affect the ability of antigen presenting cells to process or present T-cell epitopes.

The differential effects of ISS linkage ratio on T-cell versus B-cell responses allows one to selectively manipulate the immunogenicity and allergenicity of proteins. In cases in which one would like to modify proteins to produce high antibody and high T-cell responses, as is the case with many vaccine applications, conjugates with a relatively low ISS to protein ratio could be produced. In cases of allergen immunotherapy in which the objective is the induction of high T_H1 responses while avoiding IgE recognition, an ISS-linked protein with a higher ISS to protein ratio could be produced. Allergoids, chemically modified allergens typically generated by formaldehyde treatment of an antigen, have minimal IgE binding but lack sufficient immunogenicity to provide a therapeutic effect. ISS conjugation allows production of a highly immunogenic allergoid.[15,16] The high ISS to protein ratio strategy could also be applied in vaccine situations in which generation of enhancing antibodies is deleterious.

Several clinical studies have been conducted by using AIC-M as an immunotherapeutic for treatment of ragweed allergy. ISS-conjugated Amb a 1 is more than 100-fold less allergenic than licensed ragweed extract *in vivo*[17] and switches the ragweed-specific response from T_H2 toward T_H1, as evidenced by reduced IL-4, IL-5, and increased IFN-γ responses compared with placebo-treated patients.[18,19] AIC-M has been shown in phase 2 clinical trials to have efficacy in treating ragweed-specific allergic

rhinitis, reducing clinical symptoms, and the effect has so far lasted through a second allergy season.[17,19] These clinical studies further illustrate how the controlled linkage of ISS to Amb a 1 can be applied to alter the causal immune dysfunction resulting in an allergic response.

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