Conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity

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Background: Allergen immunotherapy is inconvenient and associated with the risk of anaphylaxis. Efforts to improve the safety of immunotherapy by means of chemical modification of allergens have not been successful because it greatly reduced their antigenicity. Recently, immunostimulatory DNA sequences (ISS or CpG motifs) have been shown to act as strong T_H1 response–inducing adjuvants.

Objective: We sought to determine whether conjugation of ISS to the major short ragweed allergen Amb a 1 results in enhanced immunotherapeutic potential in mice and decreased allergenicity in human subjects.

Methods: A 22-mer ISS oligodeoxynucleotide (ISS-ODN) was coupled to Amb a 1 and used for immunization of mice, rabbits, and monkeys.

Results: In mice the Amb a 1-ISS conjugate induced a T_H1 response (IFN-γ secretion), whereas Amb a 1 induced a T_H2 response (IL-5 secretion). The T_H1 response was not observed with an Amb a 1-non-ISS conjugate. Coinjection of Amb a 1 with ISS-ODN was much less effective in inducing a T_H1 response. In mice primed for a T_H2 response, injection with Amb a 1-ISS conjugate induced a de novo T_H1 response and suppressed IgE antibody formation after challenge with Amb a 1. Amb a 1-ISS conjugate induced high-titer anti-Amb a 1 IgG antibodies in rabbits and cynomolgus monkeys, whereas Amb a 1 alone or Amb a 1 coinjected with ISS-ODN did not induce a detectable response. Amb a 1-ISS conjugate was less allergenic than Amb a 1 alone, as shown by a 30-fold lower histamine release from human basophils of patients with ragweed allergy, whereas mixing ISS-ODN with Amb a 1 did not reduce histamine release.

Conclusion: Amb a 1-ISS conjugate has an enhanced T_H1-biased immunogenicity and reduced allergenicity. It may offer a more effective and safer approach for allergen immunotherapy than currently available methods. (J Allergy Clin Immunol 2000;106:124-34.)

Key words: *Allergen-ISS conjugate, immunotherapy, immunostimulatory DNA sequence, CpG motifs, basophil histamine release, ragweed allergy*

Although the efficacy of allergen immunotherapy has been proven in placebo-controlled clinical trials for allergic rhinitis and asthma, the risk of anaphylaxis, the inconvenience and discomfort of frequent dosing, and the duration of several years of therapy are all factors that limit efficacy.[1-3] Attempts at improving immunotherapy by reducing allergenicity have been paralleled by reduced immunogenicity[4,5] and did not result in an effective form of immunotherapy.

Recent understanding of the immunologic basis of allergic diseases and the mechanisms of immunotherapy revealed that immunotherapy changes the T_H2 response to allergens[6-9] to a T_H1 response,[10-13] and this is likely one of the mechanisms responsible for the beneficial and long-lasting effects. In contrast, the commonly used symptomatic treatment of allergic disorders does not have such prolonged immunomodulatory effects. The advantage of immunotherapy compared with symptomatic allergy treatments would be further enhanced by development of an immunotherapy method that could more rapidly and more strongly induce an antiallergen T_H1 response and reduce its allergenicity.

The ability to bias an immune response toward T_H1 has recently been demonstrated with bacterial DNA or immunostimulatory oligodeoxynucleotides (ISS-ODN), both of which contain CpG motifs.[14-17] ISSs cause macrophages and other antigen-presenting cells (APCs) to secrete type 1 cytokines, such as IFNs, IL-12, and IL-18, which cause naive T cells to differentiate into T_H1 cells.[15] Coimmunization with a mixture of antigens and ISSs elicits a T_H1 response[15-17] and also has been shown to be effective in animal models relevant to human allergic diseases.[18-22] Furthermore, antigen-ISS conjugates formed by an avidin-biotin bridge or chemical conjugation were reported to be very immunogenic.[23,24] Based on the crucial role of APCs in the above-mentioned system, we hypothesized that by chemical conjugation of ISS-ODN

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Abbreviations used
APC: Antigen-presenting cell
ISS: Immunostimulatory DNA sequence
ISS-ODN: Immunostimulatory oligodeoxynucleotide
mODN: Mutated (non-ISS) oligodeoxynucleotide
RW: Short ragweed

to an allergen, we would deliver the allergen and ISS-ODN to the same APC and thus minimize the dose of ISS-ODN and amplify the subsequent T_H1 response. To test this approach, ISS-ODN was chemically conjugated to the major short ragweed (RW) allergen Amb a 1 (Amb a 1-ISS conjugate) and evaluated for induction of a primary antiallergen response and effects on preexisting T_H2 responses in an animal model. The Amb a 1-ISS conjugates were also tested for reduction of allergenicity by reaction with human IgE antibodies in an ELISA test and by reduction of histamine release from basophils of patients with RW allergy.

METHODS

Purification of Amb a 1

Defatted RW pollen *(Ambrosia artemisiifolia)* was purchased from Greer Laboratories, Inc (Lenoir, NC). The Amb a 1 was isolated by using a modified method, as described by King et al.[24,25] After DEAE sepharose fast-flow anion exchange chromatography and Butyl sepharose fast-flow hydrophobic interaction chromatography, the purified Amb a 1 was concentrated by means of diafiltration to 5 mg/mL. Endotoxin levels were tested with a Limulus amoebocyte lysate analysis kit (BioWhittaker, Walkersville, Md) and found to be less than 20 ng/mL.

Oligodeoxynucleotides

The ISS 5′-TGACTGTGAA*CG*TT*CG*AGATGA phosphorothioate ODN and the mutated (non-ISS) 5′-TGACTGTGAA*CC*T-TA*G*AGATGA phosphorothioate ODN (mODN) were purchased from either Trilink BioTechnologies (San Diego, Calif) or Hybridon Specialty Products (Milford, Mass). Endotoxin levels were less than 20 ng/mL.

Preparation of Amb a 1-ISS conjugate

The purified Amb a 1 was treated with N-ethylmaleimide and activated with sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate. Residual reagents were removed by chromatography on a G-25 desalting column. 5′-Disulfide ISS-ODN was reduced with tris(2-carboxyethyl)phosphine, residual reagents were removed with chromatography on a G-25 desalting column, and the resulting 5′-Thio ISS-ODN was mixed with the activated Amb a 1 to produce the Amb a 1-ISS conjugate. The conjugate was purified by Superdex HR 200 Gel Filtration chromatography. Nonreducing SDS-PAGE, combined with Coomassie blue and DNA-specific silver staining, was used as a qualitative measure of composition of the conjugate. The method used a Novex 8% to 16% TRIS-glycine minigel at a constant current of 25 mA per gel. Conjugate bands were visualized with either Coomassie brilliant blue G-250 stain or DNA silver stain (Pharmacia DNA Silver Staining Kit).

The average number of ODNs per Amb a 1 in the conjugate was estimated by the ratio of the molar ODN content divided by the molar protein content. The ISS-ODN content was determined by using A260, and the protein content was determined by using the BCA assay. The extinction coefficient at 260 nm for 5′ ODNs was determined to be 25.6 $mg \cdot mL^{-1}cm^{-1}$. Endotoxin levels were less than 20 ng/mL, resulting in less than 1 ng in the 50 μL injected per mouse.

Immunization

Groups of 4 to 10, 4- to 6-week-old female BALB/c mice were rested a minimum of 3 days after receipt (Jackson Laboratories, Bar Harbor, Me) and were injected intradermally at the base of the tail with 50 μL of saline containing either (1) 10 μg of Amb a 1 alone, (2) a mixture of 10 μg of Amb a 1 and 7.9 μg of ISS-ODN, or (3) an Amb a 1-ISS conjugate (containing 10 μg of Amb a 1 and 7.9 μg of ISS-ODN). In some experiments Amb a 1 was also injected intraperitoneally mixed with 3 mg of alum in 500 μL of saline solution.

Mice were bled from the retro-orbital plexus after achievement of anesthesia, and serum was separated by means of centrifugation and stored at –20°C before being analyzed. Three days before being killed, the mice were injected intravenously with 5 μg of Amb a 1 to increase the frequency of antigen-specific T cells for subsequent in vitro assays.[15]

Groups of 5 New Zealand White rabbits were injected subcutaneously with either (1) 10 μg of Amb a 1, (2) 10 μg of Amb a 1 mixed with 50 μg of ISS-ODN, (3) 10 μg of Amb a 1 mixed with 500 μg of ISS-ODN, or (4) Amb a 1-ISS conjugate (containing 10 μg of Amb a 1 and 7.9 μg of ISS-ODN) each in saline solution. Rabbit sera were obtained before immunization and 2 weeks after each immunization and stored frozen at –20°C.

Groups of 4 cynomolgus monkeys were injected subcutaneously in the thigh at weeks 0, 4, and 12 with 50 μg of Amb a 1 or with Amb a 1-ISS conjugate (containing 39 μg of ISS-ODN conjugated to 50 μg of Amb a 1) each in saline solution. Sera were obtained before immunization and 2 weeks after each immunization and stored frozen at –20°C.

All animal experiments were approved by the appropriate committees on animal experimentation at the University of California San Diego, the Sidney Kimmel Cancer Center, or Sierra Biomedical, Inc (Sparks, Nev). Approval for rabbit experiments was given by Babco (Berkeley, Calif).

Antibody analyses

Mouse sera were analyzed for IgG1 and IgG2a anti-Amb a 1 antibodies by using an ELISA, as previously described.[26] The values of the test sera were calculated from a standard serum pool that was previously titrated. This standard serum pool was assigned a value in units per milliliter that was equal to the reciprocal of the highest dilution that gave an OD reading double that of the background.

IgE anti-Amb a 1 antibody titers of the mice were determined by using a modified ELISA, as previously described.[26] The modification consisted of first absorbing the serum with protein G to remove IgG anti-Amb a 1 antibodies that compete with IgE antibodies for antigen in the ELISA test. Aliquots of individual sera were added to a 50% slurry of protein G Sepharose beads (Pharmacia, Piscataway, NJ) in borate-buffered saline (pH 8.5) at a final 1:10 dilution and rotated overnight at 4°C. ELISA plates were coated with 5 μg/mL Amb a 1 in carbonate buffer (pH 9.0). Each plate contained a titration of a protein G–absorbed standard serum pool that was assigned a value in units per milliliter that was equal to the reciprocal of the highest dilution that gave an OD reading double that of the background. Units per milliliter of the test sera were calculated relative to this standard serum.

The ability of Amb a 1-ISS conjugate to bind human Amb a 1–specific IgE was measured by using a competition ELISA assay. ELISA plates were coated overnight at 4°C with Amb a 1 at 1 μg/mL. A 1:600 dilution of a high-titer human IgE anti-Amb a 1 sera was incubated with 500 ng/mL or serial 3-fold dilutions of Amb a 1 or Amb a 1-ISS conjugate for 1 hour at room temperature

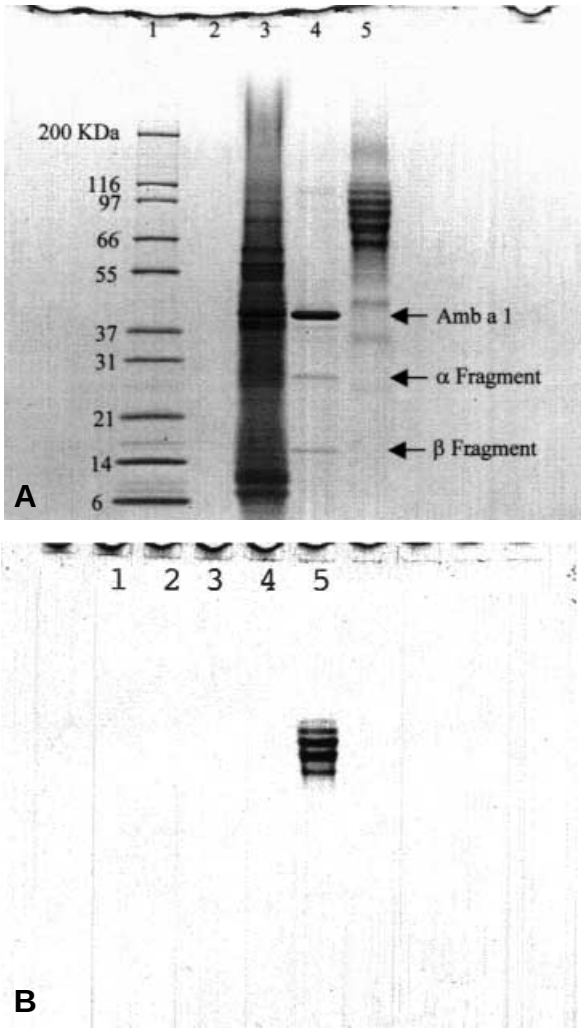

FIG 1. Nonreduced SDS-PAGE analysis of short RW extract, purified Amb a 1, and Amb a 1-ISS conjugate having an average Amb a 1/ISS-ODN molar ratio of 1:4 (gel: 4%-20%, TRIS-glycine-SDS buffer). **A,** Coomassie stain; **B,** DNA silver stain. *Lanes 1,* Molecular weight standards (Mark 12, Novex); *lanes 2,* blank; *lanes 3,* RW extract; *lanes 4,* purified Amb a 1; *lanes 5,* purified Amb a 1-ISS conjugate.

before being tested for binding to Amb a 1 in the ELISA assay by using a goat anti-human ε-chain–specific biotin conjugate (Biosource International, Camarillo, Calif) diluted 1:50.

Rabbit antisera were similarly analyzed for Amb a 1–specific IgG responses by means of ELISA. A goat anti-rabbit IgG horseradish peroxidase–conjugated antibody (Boehringer Mannheim, Indianapolis, Ind) was used to detect IgG anti-Amb a 1 antibodies.

Monkey sera were also similarly analyzed for Amb a 1–specific IgG responses by means of ELISA with a goat anti-monkey IgG horseradish peroxidase–conjugated antibody (ICN/Cappel, Costa Mesa, Calif).

Cytokine profile of splenocytes

Mice were killed by means of cervical dislocation, and their spleens were removed. Spleens were teased into single-cell suspensions, and 5×10^5 splenocytes were added to triplicate wells in a total volume of 200 μL and stimulated with either 20 μg/mL Amb a 1 protein or medium as a negative control. Cultures were incubated at 37°C in a 5% CO_2 and water-saturated atmosphere. Supernatants were harvested after 3 days and frozen at –20°C before being analyzed. The levels of IFN-γ and IL-5 were quantitated by ELISA, as previously described,[26] by using paired antibodies obtained from Pharmingen (San Diego, Calif).

Histamine release from human basophils

Venous blood was collected from volunteers with RW allergy and mixed with a solution of dextrose, EDTA, and dextran to sediment the erythrocytes, as previously described.[27] The leukocyte supernatant was used as the source of basophils. Cells were mixed with Amb a 1, Amb a 1-ISS conjugate, Amb a 1 mixed with ISS-ODN, or ISS-ODN alone and incubated for 45 minutes at 37°C with mixing every 15 minutes. The cells were centrifuged, and the histamine content of the supernatant was determined with an autoanalyzer. 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 using lysis of the cells with 2% $HClO_4$.

Statistical analyses

The differences between groups are presented as the mean ± SE. Statistical significance of differences between groups ($P < .05$) was determined by using ANOVA. The *P* values of the IgG responses of rabbits and monkeys were calculated by using nonparametric analysis.

RESULTS

Characterization of Amb a 1-ISS conjugate

Analysis of the conjugates revealed a population of 10 Amb a 1-ISS or Amb a 1-mODN conjugate bands migrating between the apparent molecular weights of 50 and 120 kd, and all were positive as determined by DNA-specific silver staining, indicating that DNA was bound to Amb a 1 (Fig 1, *A* and *B*). The Amb a 1-mODN conjugate (non-ISS) had an appearance similar to that of the Amb a 1-ISS conjugate (not shown).

The average number of ODN molecules conjugated to a single Amb a 1 molecule was estimated from the ratio of the molar ODN content to the molar Amb a 1 content, which revealed an average number of ODNs per Amb a 1 molecule of 4.

Primary immune response in mice to Amb a 1 or Amb a 1-ISS conjugate

The experimental protocol for the primary, secondary, and tertiary immunization experiments is shown in Fig 2. In the primary immunization experiment BALB/c mice were injected intradermally at the base of the tail with either Amb a 1-ISS conjugate or Amb a 1 in saline solution. Control groups received Amb a 1-mODN conjugate or a mixture of Amb a 1 and ISS-ODN (equivalent in amount to that in the conjugate). A control group of mice was also injected intraperitoneally with Amb a 1 in alum to induce a strong T_H2 response and IgE antibodies. The IgG2a, IgG1, and IgE antibody formation in these mice is shown in Fig 3. Mice immunized with Amb a 1 alone, Amb a 1-mODN conjugate, or Amb a 1 in alum formed IgG1 antibodies against Amb a 1 but no IgG2a antibodies. In contrast, mice injected with Amb a 1-ISS conju-

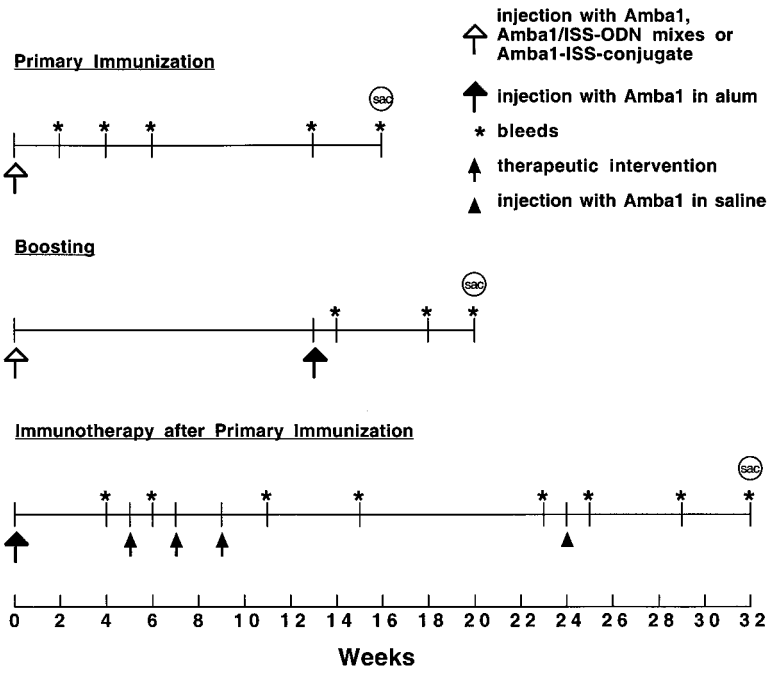

FIG 2. Experimental protocols for primary immunization, boosting with Amb a 1 in alum, and immunotherapy after primary immunization with Amb a 1 in alum and subsequent challenge with Amb a 1 in saline solution that were used for the Amb a 1–based vaccines in mice. *SAC,* Sacrifice.

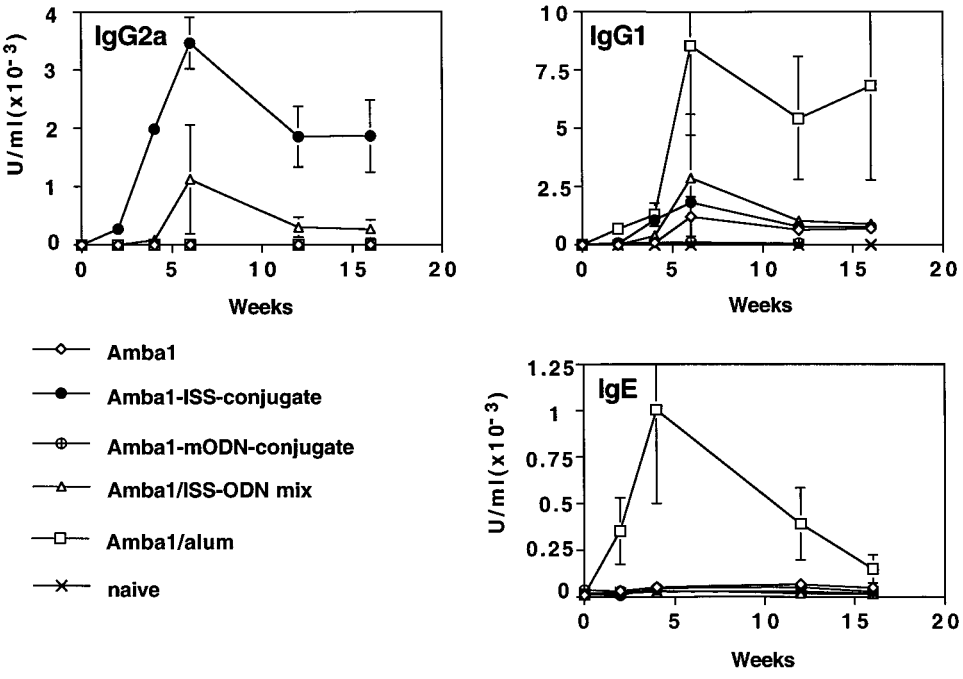

FIG 3. Antibody profile in BALB/c mice injected intradermally with 10 μg each of either Amb a 1, Amb a 1-ISS conjugate, Amb a 1 mixed with 7.9 μg of ISS-ODN (equivalent of ISS-ODN in Amb a 1-ISS conjugate), or Amb a 1-mODN conjugate. Amb a 1 in alum was injected in another group to induce a strong T_H2 response. Mean ± SE of data from 4 mice and representative data from one of 4 experiments.

TABLE I. Cytokine profile of Amb a 1–activated spleen cells from mice immunized as indicated

Antigen used for immunization	IFN-γ (pg/mL)	IL-5 (pg/mL)
Amb a 1	<10	630 ± 150
Amb a 1-ISS conjugate	8340 ± 2170*	<10*
Amb a 1-mODN conjugate	<10	500 ± 150
Amb a 1/ISS-ODN mix	170 ± 110	610 ± 280
Amb a 1/alum	490 ± 240	4410 ± 880
Saline (naive)	<10	<10

Values are means ± SE of data from 8 mice per group (killed at week 16). See Fig 2 for experimental design.
*$P < .05$ for the Amb a 1-ISS conjugate compared with the other groups.

gate responded predominantly with IgG2a antibody directed against Amb a 1. Mice injected with a mixture of Amb a 1 and ISS-ODN also generated an IgG2a antibody response; however, the titer was 3-fold ($P < .05$) lower than that observed with the conjugate. The IgG1 response to Amb a 1 was 20-fold higher than that of mice that received the conjugate. Amb a 1 injected in alum induced significantly ($P < .05$) higher titers of IgG1 and IgE antibodies than the other groups, which persisted for 16 weeks.

Cytokine profile of activated splenocytes after the primary immune response

After the last injection, mice were killed, and their spleen cells were stimulated in vitro with Amb a 1. The levels of the IFN-γ and IL-5 secreted in vitro by Amb a 1-stimulated spleen cells are shown in Table I. Spleen cells from mice immunized with Amb a 1-ISS conjugate secreted almost 50-fold more IFN-γ than cells from the mice immunized with the mixture of Amb a 1 and the ISS-ODN. IFN-γ was not detected in supernatants obtained after stimulation of splenocytes from mice injected with either Amb a 1 alone or with Amb a 1-mODN conjugate. Splenocytes from Amb a 1 in alum-injected mice secreted significantly more IL-5 than cells from the other groups (6- to 9-fold more). No IL-5 was detected from splenocytes from mice injected with Amb a 1-ISS conjugate. Control splenocytes without antigen did not have detectable levels of either IFN-γ or IL-5. The IgG2a antibody titer and IFN-γ production after primary immunization indicated that Amb a 1-ISS conjugate induced the strongest T_H1 response to Amb a 1, and therefore this modality was selected for the subsequent immunotherapy protocols.

Prevention of a T_H2 response by priming with Amb a 1-ISS conjugate

To determine whether the primary T_H1 response to Amb a 1-ISS conjugate persisted even after boosting with Amb a 1 in alum, mice were first primed with the Amb a 1-ISS conjugate and then administered a booster injection with Amb a 1 in alum (Fig 2). Control mice were primed with either (1) Amb a 1 alone, (2) Amb a 1-mODN conjugate, (3) Amb a 1 mixed with ISS-ODN, or (4) Amb a 1 in alum. As shown in Fig 4, boosting with Amb a 1 in alum caused an increase of the IgG2a titer in mice previously primed with Amb a 1-ISS conjugate, whereas it did not induce IgG2a antibodies in the Amb a 1–primed control mice. Amb a 1 in alum boosted the IgG1 response in all groups, even those injected with Amb a 1-ISS conjugate. The IgE titer rose in all mice boosted with Amb a 1 in alum; however, the response was significantly less ($P < .05$) in the mice primed with Amb a 1-ISS conjugate or primed with Amb a 1 mixed with ISS-ODN.

Cytokine profile of activated splenocytes after priming with Amb a 1-ISS conjugate and boosting with Amb a 1

The in vitro lymphokine profile in response to Amb a 1 was measured in activated spleen cells obtained 7 weeks after the booster injection of Amb a 1 in alum (Table II). Splenocytes from mice primed with Amb a 1-ISS conjugate secreted up to 5-fold higher levels of IFN-γ than did spleen cells obtained from mice primed with a mixture of Amb a 1 and ISS-ODN, Amb a 1-mODN conjugate, Amb a 1 in saline solution, or Amb a 1 in alum. The lowest IL-5 levels were in the Amb a 1-ISS conjugate–primed group, down to 15% of that obtained from the control groups. Control splenocytes cultured without antigen had neither detectable levels of IFN-γ nor IL-5. These data indicate that boosting with Amb a 1 in alum reenforced the preexisting T_H1 response in mice primed with Amb a 1-ISS conjugate and only induced a weak de novo T_H2 response in the same animals.

Effect of Amb a 1-ISS conjugate immunization on T_H2-primed mice and subsequent response to challenge with Amb a 1

Although a mouse model cannot precisely mimic the clinical situation of an allergic patient receiving allergen immunotherapy, the immune response of a T_H2-primed animal is useful in evaluating a potential therapeutic approach. For this purpose, mice were primed with Amb a 1 in alum to induce an Amb a 1–specific T_H2 response and then were injected 3 times with Amb a 1-ISS conjugate (Fig 2). Control mice received secondary injections 3 times with Amb a 1 in saline solution (analogous to immunotherapy used in human subjects), and another control group did not receive any secondary immunization. To evaluate the effect of immunotherapy in this model on subsequent allergen exposure, mice were subsequently challenged (tertiary immunization) with Amb a 1 in saline solution 15 weeks after the last injection, and the antibody response was determined. As shown in Fig 5, the IgG2a titer increased after the secondary immunization only in the group of mice injected with Amb a 1-ISS conjugate. The IgG1 titer increased in all groups after the secondary immunization, although this increase

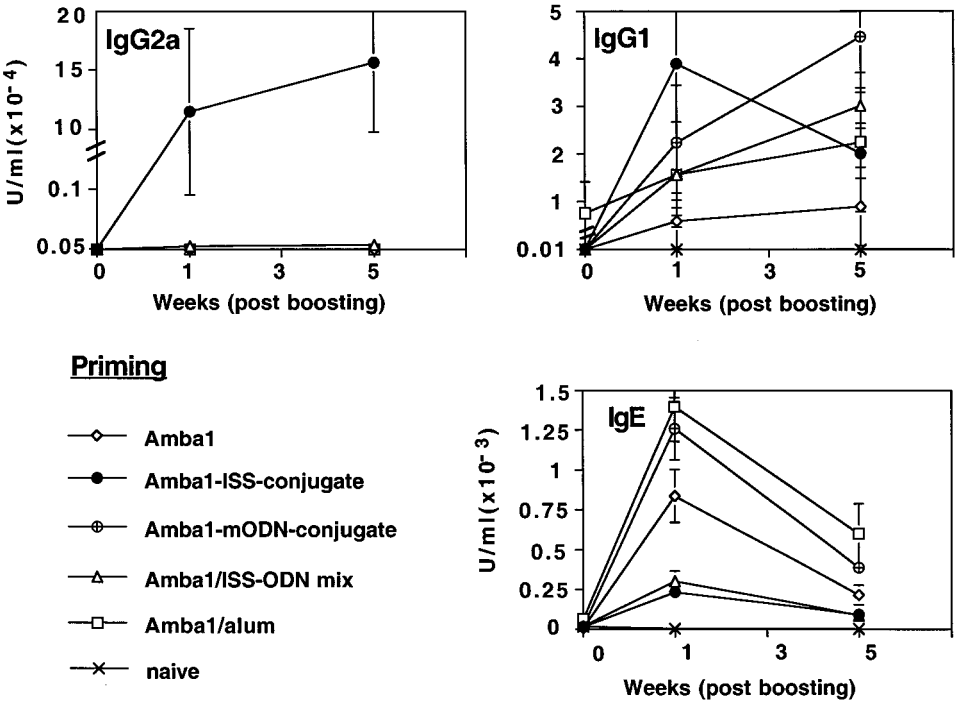

FIG 4. Antibody profile in BALB/c mice boosted intraperitoneally with 10 μg of Amb a 1 in alum after intradermal priming with 10 μg each of either Amb a 1, Amb a 1-ISS conjugate, and Amb a 1-mODN conjugate in saline solution or intraperitoneal priming with Amb a 1 in alum. Mean ± SE of 4 mice and representative data from one of two experiments.

TABLE II. Cytokine profile of Amb a 1–activated spleen cells from mice as indicated and boosted with Amb a 1/alum at week 13

Antigen used for priming	Secondary immunization	IFN-γ (pg/mL)	IL-5 (pg/mL)
Amb a 1	Amb a 1/alum	659 ± 197	840 ± 373
Amb a 1-ISS conjugate	Amb a 1/alum	4595 ± 1527*	144 ± 32*
Amb a 1-mODN conjugate	Amb a 1/alum	397 ± 34	613 ± 121
Amb a 1/ISS-ODN mix	Amb a 1/alum	788 ± 164	708 ± 93
Amb a 1/alum	Amb a 1/alum	472 ± 127	1361 ± 331
Saline (naive)	Saline	<10	<10

Values are means ± SE of spleens of 4 mice per group (killed at week 20). See Fig 2 for experimental design.
*$P < .05$ for the Amb a 1-ISS conjugate compared with the other groups.

was small and not significantly different from the prechallenge level. Secondary immunization with Amb a 1 in saline solution but not with Amb a 1-ISS conjugate increased the IgE titer. After the tertiary immunization with Amb a 1 in saline solution (rechallenge after immunotherapy), the IgG2a titer increased 4-fold in the Amb a 1-ISS conjugate–treated group, whereas it increased only slightly in the other groups. The IgE titer rose over 10-fold in the mice that did not receive secondary immunization. The small rise in the IgE titer in mice that were injected with Amb a 1-ISS conjugate during secondary immunization was significantly less ($P < .05$) than that of the untreated or Amb a 1–treated mice. Mice that were injected with Amb a 1 in saline solution showed a 1.5-fold rise in IgE; however, the rise was significantly less than that of the untreated mice.

Cytokine profile of activated splenocytes after priming with Amb a 1 in alum, immunotherapy with Amb a 1-ISS conjugate, and challenge with Amb a 1

The lymphokine profile of splenocytes from mice killed 8 weeks after the tertiary injection with Amb a 1 in saline solution is shown in Table III. Mice that received Amb a 1-ISS conjugate during the secondary immunization secreted the highest levels of IFN-γ approximately 4 times higher than that of the mice that received Amb a 1 in saline solution or alum during the secondary immunization. The IL-5 secretion was 2.5-fold lower in the mice that received a secondary immunization, either with Amb a 1-ISS conjugate or Amb a 1 compared with that of untreated mice. IFN-γ and IL-5 were not detected in

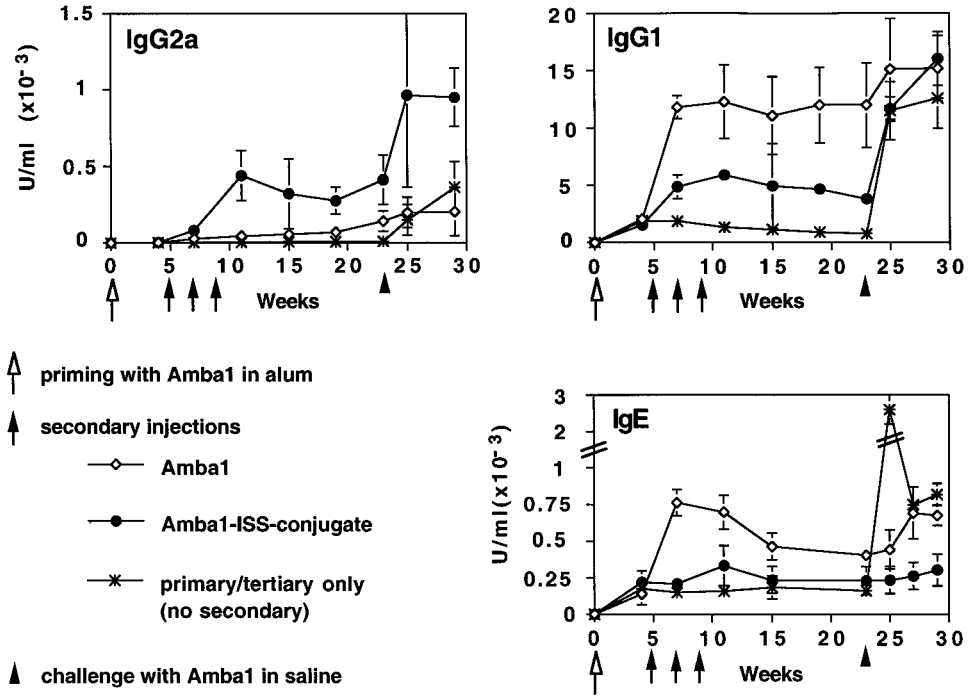

FIG 5. Effect of secondary immunization with Amb a 1 or Amb a 1-ISS conjugate on tertiary IgG2a, IgG1, or IgE antibody formation in response to challenge with 10 μg of Amb a 1 in saline solution of BALB/c mice previously primed with Amb a 1 in alum. Mean ± SE of 4 mice and representative data from one of two experiments.

TABLE III. Cytokine profile of Amb a 1–activated spleen cells of mice primed with Amb a 1 in alum and then immunized with Amb a 1-ISS conjugate or Amb a 1 alone and challenged by intraperitoneal injection of Amb a 1 in saline solution

Priming	Secondary immunization	Tertiary immunization	IFN-γ (pg/mL)	IL-5 (pg/mL)
Amb a 1/alum	Amb a 1	Amb a 1	602 ± 202	2380 ± 587†
Amb a 1/alum	Amb a 1-ISS conjugate	Amb a 1	2318 ± 101*	2584 ± 506†
Amb a 1/alum	Saline	Amb a 1	1033 ± 133	6276 ± 1332
Saline	Saline	Saline	<10	<10

Values are means ± SE of spleens of 4 mice per group (killed at week 32). See Fig 2 for experimental design.
*$P < .05$ for the group primed with Amb a 1/alum and treated with Amb a 1-ISS conjugate compared with the other groups.
†$P < .05$ of the Amb a 1 and the Amb a 1-ISS conjugate–treated groups compared with the untreated control group.

the control splenocytes that did not have antigen. The antibody response and the lymphokine profile in this set of experiments demonstrate that a secondary immunization with either Amb a 1-ISS conjugate or Amb a 1 prevents the large increase of the IgE titers and significantly reduces IL-5 secretion after re-exposure to Amb a 1. Although the absolute amount of secreted IL-5 was still substantial, treatment with Amb a 1-ISS conjugate induced very high levels of IFN-γ that provided an IFN-γ/IL-5 ratio that is likely to cause a decrease of the effects of the residual IL-5 formation.

IgG antibody response to Amb a 1, Amb a 1 mixed with ISS, or Amb a 1-ISS conjugate in rabbits and cynomolgus monkeys

Rabbits were immunized with Amb a 1, Amb a 1 mixed with 50 or 500 μg of ISS-ODN, or Amb a 1-ISS conjugate (Fig 6, *A*). All groups showed low antibody titers in preimmunization bleeds, ranging from 263 to 495 U/mL. No increase in anti-Amb a 1 IgG titers was detected after two immunizations with Amb a 1 mixed with 50 or 500 μg of ISS-ODN. In contrast, two immunizations with Amb a 1-ISS conjugate increased the mean anti-Amb a 1 IgG titers from 495 to 106,000 U/mL ($P < .05$).

Cynomolgus monkeys were immunized with Amb a 1 or Amb a 1-ISS conjugate (Fig 6, *B*). All the animals had some background anti-Amb a 1 IgG titers (prebleed samples), ranging from 350 to 450 U/mL. No increases in the anti-Amb a 1 IgG titers were detected after one, two, or three immunizations with Amb a 1. In contrast, two immunizations with Amb a 1-ISS conjugate enhanced the mean anti-Amb a 1 IgG antibody titer 18-fold (449 vs 8040 U/mL, $P < .05$). No IgE anti-Amb a 1 antibodies were detectable in any of the monkey sera before or after immunization (data not shown).

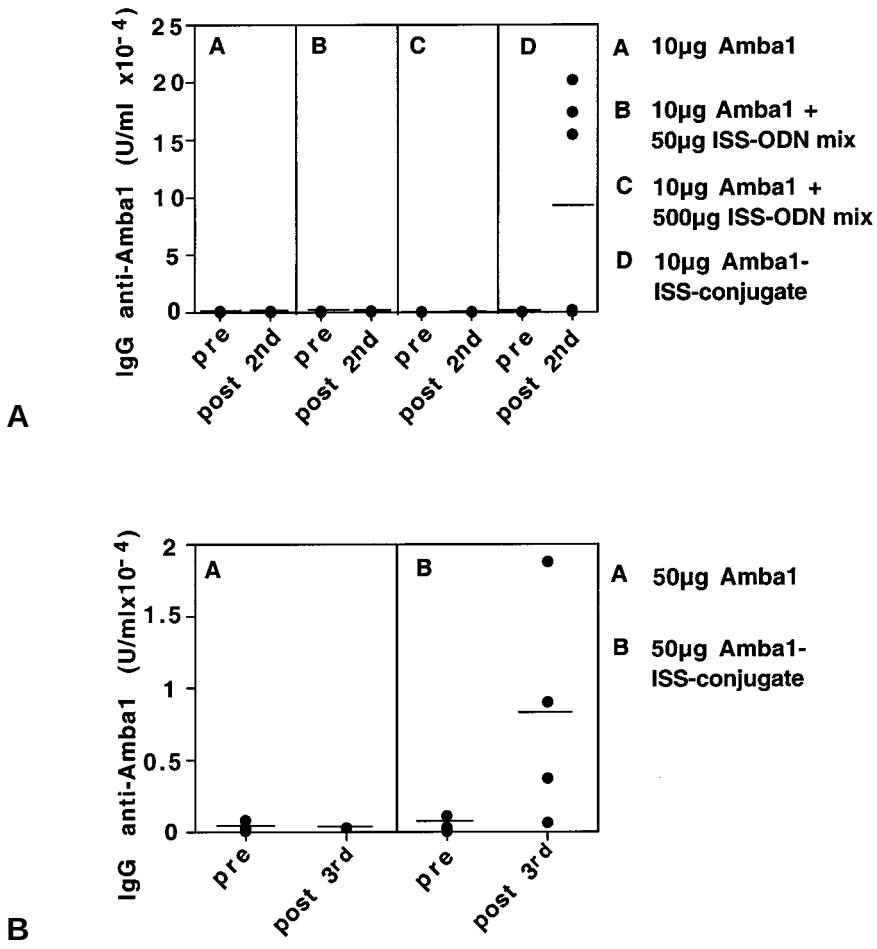

FIG 6. A, IgG antibody titers of rabbits injected with either 10 μg of Amb a 1, 10 μg of Amb a 1-ISS conjugate, or 10 μg of Amb a 1 mixed with either 50 or 500 μg of ISS-ODN. **B,** IgG antibody titers of cynomolgus monkeys injected with 50 μg of Amb a 1 or 50 μg of Amb a 1-ISS conjugate. *Circles* represent the titer of an individual animal, and the *bars* represent the mean values.

Reaction with human IgE antibodies and histamine release by human basophils from patients allergic to RW induced by Amb a 1 or Amb a 1-ISS conjugate

To determine whether the chemical conjugation of ISS-ODN to Amb a 1 diminished the ability of the Amb a 1 to react with human IgE anti-Amb a 1 antibodies, the conjugate was tested in an ELISA assay for its inhibition of the reactivity of an IgE anti-Amb a 1–containing sera with Amb a 1. As shown in Fig 7, the Amb a 1-ISS conjugate reacted less well with human IgE anti-Amb a 1 antibodies than did Amb a 1. An approximate 3-fold higher concentration of Amb a 1-ISS conjugate was necessary to achieve 50% inhibition compared with native Amb a 1. To determine whether the conjugation of ISS-ODN to Amb a 1 changed its allergenic properties, basophils were obtained from 4 patients allergic to RW, and histamine release was assessed in vitro after stimulation with Amb a 1 or Amb a 1-ISS conjugate. As shown in Fig 8, on average a 30-fold higher concentration of Amb a 1-ISS conjugate was required to induce 50% of maximum histamine release in comparison with the histamine release induced by Amb a 1 alone. The histamine release induced by Amb a 1-mODN conjugate was also about 30-fold lower than that induced by Amb a 1 (data not shown). ISS-ODN alone did not induce histamine release nor did it decrease histamine release induced by native Amb a 1.

DISCUSSION

This study demonstrates that chemical conjugation of an ISS-ODN to the major RW allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity. Mice immunized with Amb a 1-ISS conjugate generated high levels of IgG2a antibodies and IFN-γ–secreting cells, whereas native Amb a 1 induced IgG1 and IgE antibodies and IL-5–secreting T cells. Mice injected with Amb a 1-ISS conjugate also formed IgG1 antibodies; however, IgG1 formation is not always restricted to T_H2 responses. The observed secreted

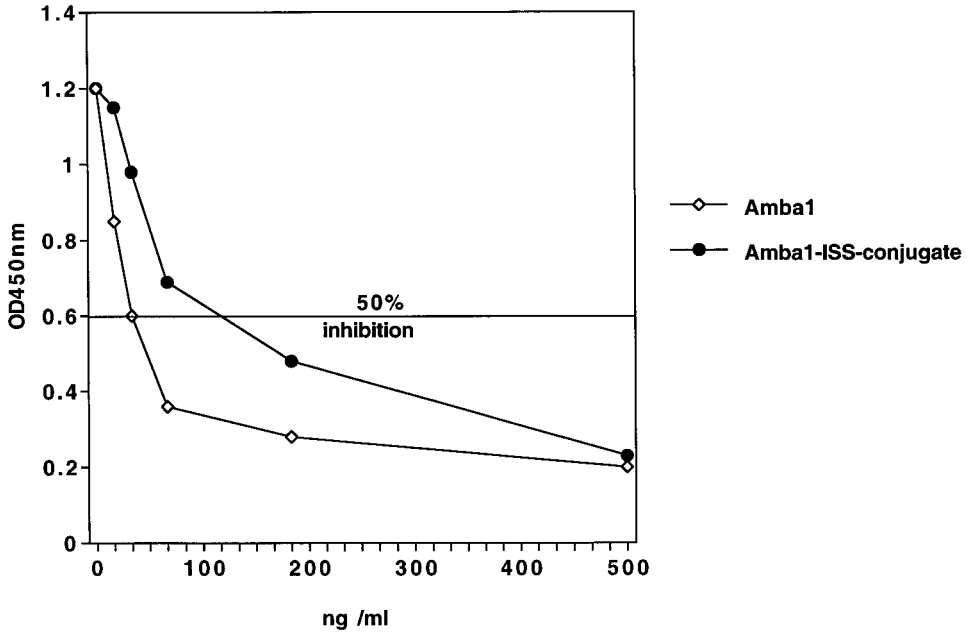

FIG 7. Inhibition of human IgE binding of a high IgE titer serum from a patient allergic to RW to Amb a 1 by Amb a 1-ISS conjugates in an ELISA inhibition test. Data shown are from one of two experiments that gave similar results. Each data point represents the mean of duplicate analyses. The variability of duplicate wells was less than 20%, and the differences between the means of the first three inhibition concentrations were statistically significant ($P < .05$).

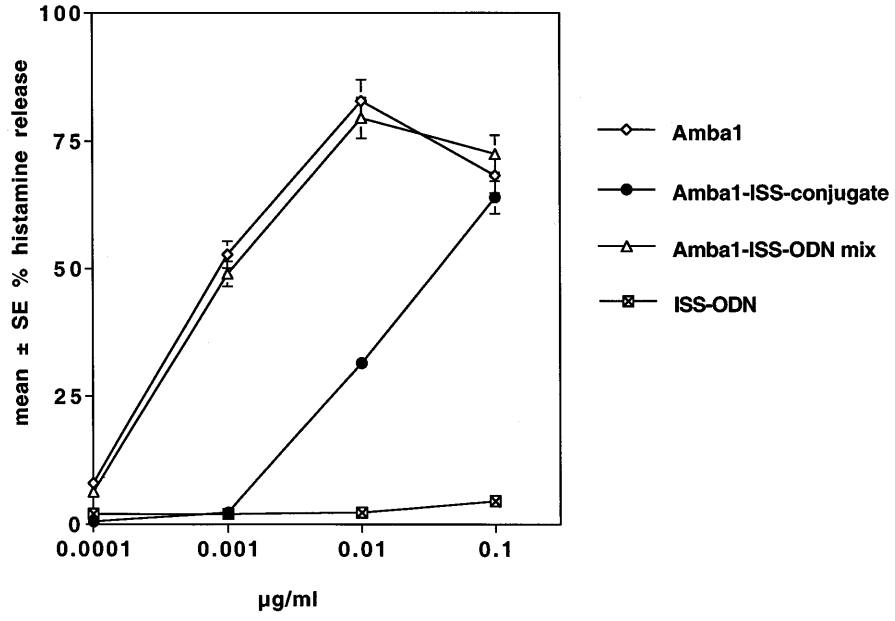

FIG 8. Histamine release from human basophils induced by either Amb a 1, Amb a 1-ISS conjugate, Amb a 1 mixed with ISS-ODN at a dose equivalent to that conjugated to 10 µg of Amb a 1, or ISS-ODN alone. Mean ± SE histamine release from basophils of 4 different donors with RW allergy is shown.

lymphokines were typical for a T_H1 and T_H2 immune response, respectively.[28] Amb a 1 conjugated to a non-ISS control ODN (mODN) in which the two CG dinucleotides were mutated to CC and AG did not have this effect. The increased immunogenicity of the Amb a 1-ISS conjugate is different from the modified allergens (allergoids) that were used in the past[4,5] because allergoids lost most of their immunogenicity after chemical modification. Moreover,

and clinically most importantly, a preexisting Amb a 1–specific T_H2 response did not prevent induction of a de novo allergen-specific T_H1 response to immunization with Amb a 1-ISS conjugate and suppressed IgE antibody formation to a tertiary injection of Amb a 1. Furthermore, as judged by in vitro histamine release, the conjugate was less allergenic: about a 30-fold higher concentration of conjugate was necessary to induce the same amount of histamine release as that induced by Amb a 1. These properties of allergen-ISS conjugates indicate that they offer a novel type of modified allergen that may provide an effective and safer approach to immunotherapy in human subjects than that presently available.

The effect of immunotherapy on downregulation of an allergic response in an animal model has to our knowledge not been thoroughly investigated in the past, presumably because it was used as an established therapy for allergic disorders in human subjects long before the immunologic mechanisms underlying allergic disorders were discovered. Because we prepared the Amb a 1-ISS conjugate for a more effective and safer immunotherapy, we compared the effect of Amb a 1-ISS conjugate immunotherapy with that of Amb a 1 in mice primed with Amb a 1 in alum to induce a preexisting T_H2 response. Perhaps not too surprisingly, both the Amb a 1-ISS conjugate and Amb a 1 injections prevented a large increase of the IgE antibody formation on a tertiary challenge with Amb a 1, and both caused a significant decrease of the IL-5 secretion by splenic T cells compared with untreated mice. These data parallel the clinical observations of the blunting of the IgE response of immunotherapy-treated patients during the ragweed pollen season and data showing that immunotherapy could change allergen-specific T cells from a T_H2- to a T_H1-like type.[10-13] However, in mice the Amb a 1-ISS conjugate therapy had a qualitatively much stronger beneficial effect than the Amb a 1 injections. Amb a 1-ISS conjugate treatment induced much higher titers of IgG2a antibodies than Amb a 1 injections, suggesting a stronger T_H1 response. Although the T cells from the Amb a 1-ISS conjugate– and Amb a 1–treated mice still secreted substantial amounts of IL-5, the high IFN-γ secretion by the conjugate-treated mice resulted in an IFN-γ/IL-5 ratio that is likely to decrease any residual IL-5 effects. Second, Amb a 1-ISS conjugate injections suppressed the IgE antibody formation more strongly than the Amb a 1 injections. Whether Amb a 1-ISS conjugate immunotherapy is more efficient than classical immunotherapy in allergic patients remains to be investigated.

Coinjection of Amb a 1 with ISS-ODN also induced a T_H1 response in mice; however, it was weaker than that induced with the corresponding conjugate. Coinjection of mice with a mixture of Amb a 1 and ISS-ODN induced a lower IgG2a titer, and T cells from these mice secreted 10-fold less IFN-γ than T cells from mice immunized with a conjugate that contained an equivalent amount of Amb a 1 and ISS-ODN. The enhanced immunogenicity of Amb a 1-ISS conjugates was even more dramatic in rabbits and monkeys. These species formed high titers of IgG antibodies to the conjugate but failed to respond to the mixture or antigen alone. These findings indicate that conjugation of ISS-ODN to antigens may be crucial for successful induction of immune responses to weak antigens or very low doses of antigen, as is used in allergen immunotherapy.

As noted, the increased immunogenicity of Amb a 1-ISS conjugate was not limited to murine species. Both rabbits and cynomolgus monkeys generated a significantly enhanced IgG antibody response after injection with the conjugate. This response was not observed after injection with either Amb a 1 alone (rabbits and monkeys) or after injection with a mixture of Amb a 1 and ISS-ODN (rabbits). These results are the first to report in vivo immunostimulatory properties in mice, rabbits, and nonhuman primates by the same ISS-ODN.

The mechanisms by which allergen-ISS conjugate induces a T_H1 response are not yet fully explored. ISS-ODNs were shown to activate components of the innate immune system that result in a subsequent T_H1 response.[14-17] In particular, ISS-ODN induces the secretion of type 1 cytokines[15-17,29] and induces a distinct costimulatory molecule profile on APCs.[30] Allergen-ISS conjugate allows for the codelivery of antigen and ISS to the same cell, such as an APC, and thereby optimizes the immunomodulatory effects of ISS with antigen processing. In contrast, the coinjection of Amb a 1 with ISS-ODN may result in the in vivo dissociation of the ISS from the coinjected antigen, resulting in a less efficient ISS effect on antigen processing and a weaker T_H1 response.

Subcutaneous injection of allergen, as it is performed in current standard immunotherapy, carries the risk of inducing anaphylactic reactions.[13] These adverse reactions severely limit the tolerated doses and are one of the major reasons why immunotherapy is used less today than it has been in the past. Amb a 1-ISS conjugates were less reactive with human IgE anti-Amb a 1 antibodies and required 30-fold more conjugate on a molar basis than Amb a 1 to release the same amount of histamine from basophils of patients allergic to RW. The reasons for the decreased histamine release by the conjugates are not yet fully understood. Steric hindrance or chemical alteration of the allergenic epitopes by the ISS-ODN coupled to the Amb a 1 may be one of the reasons. Should this property of allergen-ISS conjugate also result in a lower anaphylactic potential in vivo, patients might be safely injected with higher doses of allergen-ISS conjugate compared with unmodified allergen alone.

In summary, these studies demonstrate that chemical conjugation of ISS-ODN to allergen confers on the allergen two new properties that make it an excellent candidate for human immunotherapy. First, allergen-ISS conjugate induces strong T_H1 and high IgG responses, even in the face of an ongoing T_H2 response. The induction of a rapid and strong allergen-specific IFN-γ release (ie, a T_H1 response), as well as the IgG antibody formation to injections of low doses of allergen, are both desired properties that would enhance the beneficial response to an

immunotherapeutic agent. Second, the markedly lower histamine release from basophils than allergen or allergen mixed with ISS should portend a much lower risk of anaphylaxis or serious adverse events to immunotherapy. This combination of enhanced immunogenicity and reduced allergenicity is encouraging for the development of allergen-ISS conjugates as a novel and efficacious mode of allergen immunotherapy.

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