

Optimized conjugation ratios lead to allergen immunostimulatory oligodeoxynucleotide conjugates with retained immunogenicity and minimal anaphylactogenicity

Anthony A. Horner, MD,^{a,d} Kenji Takabayashi, PhD,^{a,d} Lucinda Beck, BS,^{a,d} Bhavya Sharma, PhD,^e Jose Zubeldia, MD, PhD,^a Stephen Baird, MD,^c Stephan Tuck, PhD,^f Lev Libet, BA,^a Hans L. Spiegelberg, MD,^b Fu-Tong Liu, MD, PhD,^e and Eyal Raz, MD^{a,d} La Jolla, San Diego, and Berkeley, Calif

Background: Immunotherapy has gradually fallen out of favor for the treatment of many allergic diseases because of the overall convenience, safety, and efficacy of medications. However, investigations suggest that allergen/immunostimulatory sequence oligodeoxynucleotide (ISS-ODN) conjugates (AICs) might have improved safety and efficacy compared with allergen extracts.

Objective: We determined whether changes in the ISS-ODN conjugation ratio would effect the immunogenicity and allergenicity of AIC.

Methods: Immunogenicity was determined by means of AIC vaccination of mice, followed by analysis of antigen-specific antibody and cytokine responses. The allergenicity of AIC was determined in mast cell release studies and in murine models of anaphylaxis and the Arthus reaction.

Results: AIC induced a stronger immune response than allergen alone or allergen mixed with ISS-ODN, but higher-level ISS-ODN conjugation reduced its immunogenicity modestly. In mast cell degranulation studies AIC was approximately 100-fold less allergenic than native allergen, with stepwise increases in the ODN conjugation ratio leading to stepwise decreases in allergenicity. In anaphylaxis studies death rates were reduced from 100% with native allergen challenge to as low as 0% with high-ratio ISS-ODN AIC challenge. Similar results were obtained in an Arthus reaction model.

Conclusion: These investigations establish that AIC is both significantly more immunogenic and less allergenic than native allergens and the techniques used might have further utility for the standardization and optimization of AIC formulations

for use in allergic patients. (*J Allergy Clin Immunol* 2002;110:413-20.)

Key words: Immunostimulatory DNA, CpG motif, immunotherapy, anaphylaxis

Allergen immunotherapy was initially reported to be effective by Noon¹ in 1911, and for many years, it was the first-line treatment for allergic diseases.² However, medications have gradually become the major source of therapy for allergic patients, in part because of the slow response rate, patient inconvenience, and risk of allergic and sometimes life-threatening allergic reactions associated with immunotherapy.²⁻⁴ Furthermore, although immunotherapy is highly effective for more than 90% of patients with anaphylactic hypersensitivities to stinging insects, immunotherapy appears to be somewhat less effective for respiratory allergies, and because of the risk of anaphylaxis, immunotherapy is presently contraindicated for the treatment of food allergies.^{2,5-9} There have been many efforts to improve the safety and effectiveness of immunotherapy over the years. Increasing allergen doses improve immunogenicity and effectiveness, but allergic symptoms with high-dose immunotherapy are generally more severe than symptoms in untreated individuals during natural exposure.^{2,10} Alternatively, formalin-denatured, polyethylene glycol-conjugated, and other modified allergens with reduced allergenicity have been developed.^{2,11,12} Unfortunately, despite their lower allergenicity, these modified allergens have been consistently shown to be less immunogenic and less clinically effective than allergen extracts for the treatment of allergic diseases.

Immunostimulatory sequence oligodeoxynucleotides (ISS-ODNs), also termed CpG motifs because of this shared dinucleotide, are potent inducers of innate, T_H1, and mucosa immune responses.¹³⁻¹⁷ Further investigations have established that ISS-ODN-based therapies can prevent T_H2-biased immune responses, divert T_H2-biased immune responses, or both and attenuate allergic hypersensitivity responses in several animal models.¹⁸ Park et al¹⁹ recently demonstrated that ISS-ODN sequences mod-

From the Departments of ^aMedicine, ^bPediatrics, and ^cPathology and ^dThe Sam and Rose Stein Institute for Aging, University of California, San Diego, La Jolla; ^eLa Jolla Institute of Allergy and Immunology, San Diego; and ^fDynavax Technologies Corporation, Berkeley.

Supported in part by grants AI01490 and AI40682 from the National Institutes of Health and by a grant from Dynavax Technologies Corporation.

Received for publication April 9, 2002; revised May 13, 2002; accepted for publication May 22, 2002.

Reprint requests: Eyal Raz, MD, University of California San Diego, 9500 Gilman Dr, La Jolla, CA 92093-0663.

© 2002 Mosby, Inc. All rights reserved.

0091-6749/2002 \$35.00 + 0 1/83/126660

doi:10.1067/mai.2002.126660

Abbreviations used

AIC:	Allergen/ISS-ODN conjugate
BMMC:	Bone marrow–derived mast cell
(H):	High ratio
ISS-ODN:	Immunostimulatory oligodeoxynucleotide
(L):	Low ratio
(M):	Medium ratio
M-ODN:	Mutated oligodeoxynucleotide
ODN:	Oligodeoxynucleotide
OVA:	Ovalbumin
RBL:	Rat basophilic leukemia

ified by the attachment of 3' guanine hexamers have improved antiallergic activities relative to unmodified ISS-ODN sequences. We and others have developed another ISS-ODN–based reagent in which antigen (allergen) is physically conjugated to ISS-ODN (ie, allergen/ISS-ODN conjugate [AIC]). Several laboratories have demonstrated that ISS-ODN conjugation leads to ODN-facilitated uptake of antigen, resulting in improved immunogenicity of AIC relative to antigen mixed with ISS-ODN, and ODN conjugation has been further shown to mask Ig-binding epitopes on the allergen.^{14,20-22}

In this series of investigations, we considered how the ISS-ODN conjugation ratio and sequence affect the immunogenic and allergenic properties of AIC. We speculated that although ISS-ODNs conjugated to allergen would provide adjuvant activity and hence improve immunogenicity, the bound and negatively charged ODNs might also mask epitopes important for B- and T-cell responses. These antithetical considerations would suggest that an optimal ISS-ODN/allergen ratio exists for maximal AIC immunogenicity. We further hypothesized that the ODN/allergen conjugation ratio of AIC formulations would be inversely correlated with their allergenicity because of more efficient masking of Ig-binding epitopes at higher ODN conjugation ratios but that allergenicity would not be linked to the sequence of the ODN used for conjugation. The present studies support these hypotheses and highlight the improved immunogenicity and reduced allergenicity of AIC compared with allergen, either alone or mixed with ISS-ODNs.

METHODS**Mice**

Six- to 8-week-old female BALB/c mice were used in all experiments. They were purchased from Jackson Laboratories (Bar Harbor, Me) and maintained in the University of California, San Diego, Animal Facility, which is certified by the American Association for the Accreditation of Laboratory Animal Care. All experiments were approved by the animal care committee at our institution.

Reagents

Ovalbumin (OVA) was purchased from Calbiochem (San Diego, Calif), and FITC-labeled OVA was obtained from Molecular Probes (Eugene, Ore). Phosphorothioate ISS-ODN (5'-TGACTGTGAACGTTTCGAGATGA-3') and mutated ODN (M-ODN; 5'-TGACTGTGAAGGTTAGAGATGA-3') were synthesized by

Trilink Biotechnologies (San Diego, Calif). The ISS-ODNs and M-ODNs selected for study have previously been shown to have high and low immunostimulatory activity, respectively.^{20,21} To produce OVA conjugated to ISS-ODN (OVA/ISS-ODN) and M-ODN (OVA/M-ODN), maleimido groups were introduced onto exposed lysines of the OVA molecule by means of incubation with a 20 molar excess of sulfo-succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (Pierce Chemicals, Rockford, Ill) for 2 hours, followed by purification on a NAP-25 Column (Amersham-Pharmacia, Uppsala, Sweden). A 5' thiol group was added to the ODNs by means of incubation with 20 μ mol/L tricarboxyethylphosphine (Pierce Chemicals), and activated ODNs were subsequently purified on a NAP-10 column. Maleimido-modified OVA and thiol-activated ODNs were then incubated together overnight at room temperature. Crude OVA/ISS-ODN and OVA/M-ODN conjugate mixtures were then loaded on a Superdex 200 (10:30) gel filtration column attached to fast protein liquid chromatography to obtain a range of OVA/ODN conjugation ratio formulations (low ratio [L], 1:2.5; medium ratio [M], 1:3.5; and high ratio [H], 1:5.5). For all experiments described in this article, OVA, OVA/ISS-ODN, and OVA/M-ODN doses were standardized on the basis of OVA content. Free ISS-ODN was used with native OVA in doses corresponding to the amount in OVA/ISS-ODN (H) at the same OVA dose.

A pool of high-titer anti-OVA hyperimmune serum was made by means of repeated intraperitoneal sensitization of mice with OVA (25 μ g) and alum (1 mg). Anti-OVA IgE-enriched serum was made by means of IgG removal with protein G–conjugated beads, as previously described.²³ Anti-OVA IgG-enriched serum was made by means of heat inactivation of IgE at 56°C for 3 hours.

Immunization, sensitization, anaphylaxis, and passive Arthus protocols

Mice were intradermally immunized with 10 μ g of OVA, OVA/ISS-ODN (L or H), OVA/M-ODN (M), or OVA mixed with 10 μ g of ISS-ODN. For the anaphylactic challenge studies, mice were sensitized with 2 weekly intraperitoneal injections of OVA (100 μ g), alum (1 mg), and pertussis toxin (100 ng; Sigma, St Louis, Mo). Three weeks after the last sensitization, mice were intravenously challenged with 50 μ g of OVA alone, OVA mixed with 50 μ g of ISS-ODN or with 50 μ g of OVA/ISS-ODN (L or H), or OVA/M-ODN (M). A subset of mice was bled 2 minutes after challenge for plasma histamine determinations, as previously described.²³ For passive Arthus studies, OVA hyperimmune serum (10 μ L) was mixed with 2 μ g of OVA alone or OVA plus ISS-ODN (2 μ g) or with 2 μ g of OVA/ISS-ODN (L or H) or OVA/M-ODN (M) in a final volume of 50 μ L. These mixtures were intradermally injected into the tail base of naive mice; tails were harvested at 6 hours, fixed, cut into 1-mm disks sampling the length of the injected area, mounted in paraffin, and finally stained with hematoxylin and eosin or stained immunohistochemically to detect complement (C3) deposition. For each mouse, the tail section with maximal inflammatory cell infiltrate and complement deposition was scored on a 4-point scale by a blinded observer, and mean scores for each injection group were calculated.

Antibody and cytokine analysis

Sera were obtained 6 weeks after immunization for ELISA analysis. The mice were killed, and OVA-specific IgG2a and IgG1 levels and splenocyte cytokine responses were measured by means of standard methods.²³

Ig-binding inhibition assays

IgE- and IgG-binding inhibition assays were performed with OVA hyperimmune IgE-enriched (1:100 dilution) or IgG-enriched (1:1000 dilution) serum mixed 1:2 with 2-fold serial dilutions of

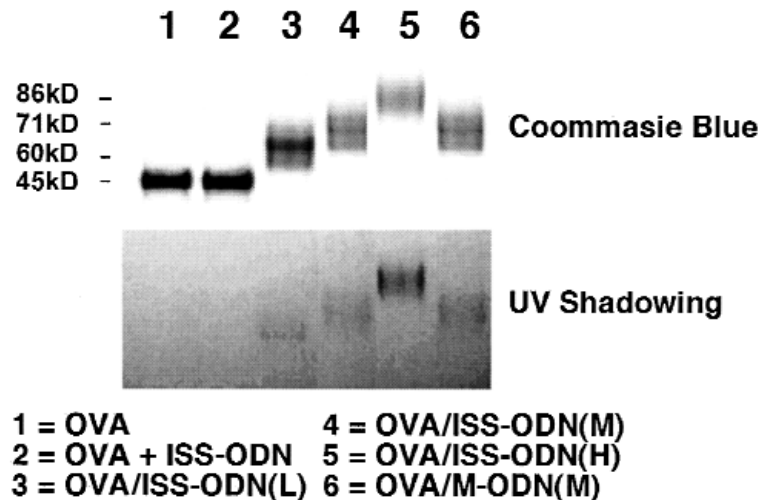


FIG 1. Coomassie blue staining and UV shadowing of OVA/ODN conjugates. Equivalent amounts of OVA, OVA plus ISS-ODN, and the various ODN conjugates (on the basis of OVA content) were examined on a nonreduced SDS-PAGE gel. Coomassie blue was used to stain protein, and UV shadowing was used to detect ODN. As reflected in *lane 2*, ODNs did not physically associate with OVA without chemical linkage. The increases in molecular weight associated with OVA/ISS-ODN (L), OVA/ISS-ODN (M), OVA/ISS-ODN (H), and OVA/M-ODN (M) correspond to mean OVA/ODN molar binding ratios of 1:2.5, 1:3.5, 1:5.5, and 1:3.5, respectively.

TABLE I. OVA/ISS-ODN (L) and OVA/ISS-ODN (H) are more immunogenic than OVA plus ISS-ODN

Immunization	IgG2a (U/mL)	IgG1a (U/mL)	IFN- γ (pg/mL)	IL-10 (pg/mL)
OVA	193 $\pm$ 79	1020 $\pm$ 608	194 $\pm$ 75	<20
OVA + ISS-ODN	596 $\pm$ 113	2330 $\pm$ 743	409 $\pm$ 98	<20
OVA/ISS-ODN (L)	8124 $\pm$ 1408*	2013 $\pm$ 1238	1385 $\pm$ 220*	748 $\pm$ 543*
OVA/ISS-ODN (H)	4840 $\pm$ 2075*	1157 $\pm$ 379	989 $\pm$ 151*	378 $\pm$ 143*
OVA/M-ODN (M)	194 $\pm$ 80	1264 $\pm$ 428	89 $\pm$ 47	<20

Mice ($n = 4/\text{group}$) received a single intradermal immunization with OVA, OVA plus ISS-ODN, or ODN-conjugated OVA. Free ISS-ODN was delivered at a dose corresponding to the ISS-ODN content of OVA/ISS-ODN (H). Antigen-specific serum antibody and splenocyte cytokine responses were determined 6 weeks later by means of ELISA. Results are presented as means $\pm$ SE and are representative of 3 separate experiments. * $P < .001$ relative to OVA vaccination.

OVA, OVA plus ISS-ODN, OVA/ISS-ODN (M), or OVA/M-ODN (M). The mixtures were incubated for 1 hour at 37°C and added to 6-well plates previously coated with OVA (5 $\mu\text{g/mL}$). After an overnight incubation at 4°C, the plates were washed, and antibodies to either mouse IgE (biotinylated; Pharmingen, San Diego, Calif) or IgG (AP conjugated; Southern Biotechnology Associates, Birmingham, Ala) were added to the plates and were subsequently incubated with reagents to detect plate-bound Ig, according to the manufacturer's recommendations.

Flow cytometric assays

Rat basophilic leukemia (RBL) cells and J774 cells (ATTC, Rockville, Md) were incubated for 1 hour with IgE- or IgG-enriched OVA hyperimmune serum (1:20 dilution). The cells were then incubated for 30 minutes with FITC-labeled OVA or FITC-labeled OVA/ISS-ODN (M) or OVA/M-ODN (M) synthesized from FITC-labeled OVA (all reagents at 100 ng/mL). For the last incubation, an irrelevant ODN was added at 5 $\mu\text{g/mL}$ to saturate Ig-independent, DNA-dependent binding of the FITC-labeled OVA/ODN conjugates. Analysis of cells was conducted with a FACSCalibur flow cytometry unit. Twenty thousand cell events were recorded per sample, and results were quantitated by means of mean fluorescence intensity ratio:

Mean fluorescence intensity in the presence of cellular preincubation with OVA hyperimmune sera/Mean fluorescence intensity in the absence of cellular preincubation with OVA hyperimmune sera.

In vitro mast cell release assay

Bone marrow-derived mast cells (BMMCs) were prepared, and degranulation was assessed as previously described.²⁴ Briefly, BMMCs ($2 \times 10^6/\text{mL}$) were sensitized overnight with a 1:100 dilution of high-titer anti-OVA IgE serum. The next day, BMMCs were washed and stimulated with OVA, OVA plus ISS-ODN, OVA/ISS-ODN (L or H), or OVA/M-ODN (M). β -Hexosaminidase activity in the supernatant and the pellet were then determined by standard means.²⁴

Statistical considerations

Statistical analyses was conducted with Statview software. Two-tailed unpaired Student t tests were conducted on all data except dose-release curves for binding inhibition and β -hexosaminidase release (ANOVA) and anaphylaxis challenge survival (Fisher exact test).

RESULTS

Relative molar ratio of ISS-ODN to OVA modestly reduces the immunogenicity of OVA/ISS-ODN

We synthesized low-ratio (1:2.5), medium-ratio (1:3.5), and high-ratio (1:5.5) OVA/ISS-ODN and medium-ratio OVA/M-ODN (1:3.5; Fig 1). Initially, we determined the relative immunogenic capacity of OVA/ISS-ODN (L) and OVA/ISS-ODN (H). Mice receiving a

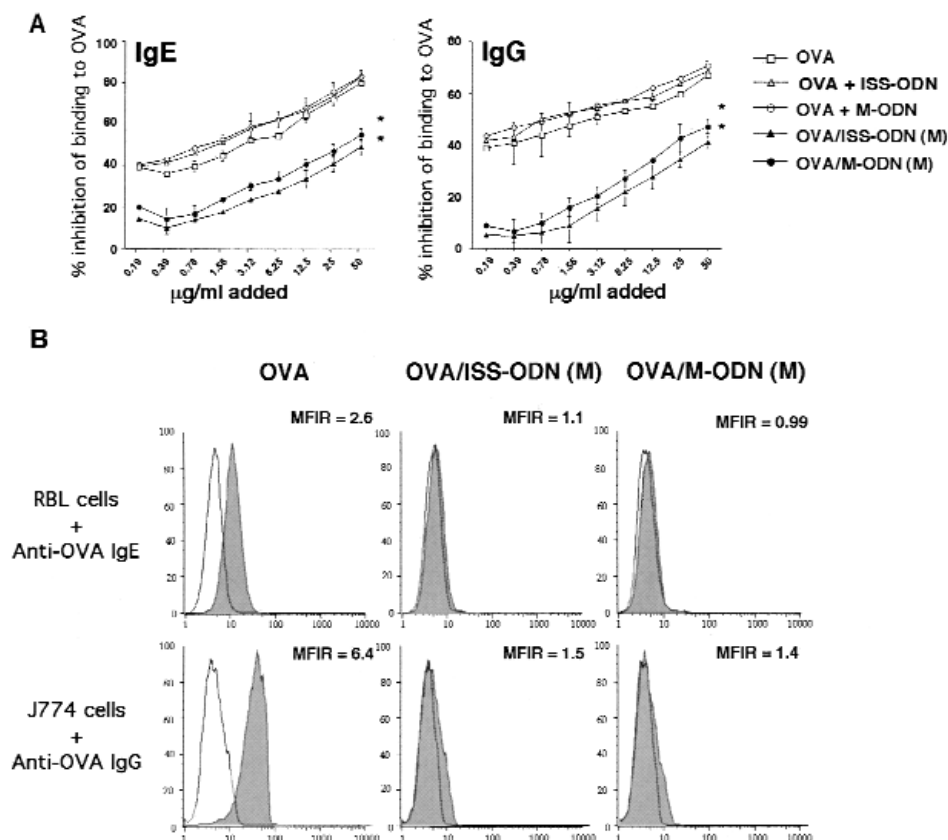


FIG 2. ODN conjugation masks OVA-specific Ig-binding epitopes. **A**, OVA, OVA plus ODN, and OVA/ODN conjugates were tested for Ig-binding inhibition, as described in the "Methods" section, and results are representative of 3 separate experiments (* $P < .001$ for dose inhibition curves relative to OVA). **B**, RBL and J774 cells were sensitized with OVA-specific IgE and IgG, respectively. Then sensitized and unsensitized cells were incubated with FITC-conjugated OVA, OVA/ISS-ODN (M), or OVA/M-ODN (M) and analyzed by means of flow cytometry. Histograms for sensitized cells are shaded, and histograms for unsensitized cells are not. The mean fluorescence intensity ratio (MFIR) was used to evaluate Ig-dependent binding of the FITC-conjugated reagents. Experiments were repeated 3 times with similar results.

single intradermal immunization with OVA/ISS-ODN (L or H) had a significantly stronger IgG2a response compared with that of mice immunized with OVA plus ISS-ODN, OVA/M-ODN (M), or OVA alone. In fact, the IgG2a response was 42- and 13-fold higher with OVA/ISS-ODN (L) versus OVA or OVA/ISS-ODN coimmunization, respectively (Table I). However, in 3 separate experiments OVA/ISS-ODN (L) induced around a 2-fold higher IgG2a response than OVA/ISS-ODN (H). The same trend was seen in separate experiments with 2 immunizations (data not shown), but differences with either 1 or 2 immunizations did not reach statistical significance. In contrast to IgG2a levels, IgG1 levels were similar for all vaccination groups.

We also evaluated the splenocyte cytokine response of immunized mice. Although antigen-specific IL-4, IL-5, and IL-13 were not detected in culture supernatants (data not shown), IFN- γ and IL-10 production was significantly greater for splenocytes derived from AIC-immunized than from control-immunized mice (Table I). However, consistent with the IgG2a response, OVA/ISS-ODN (L) tended to induce a stronger cytokine response than

OVA/ISS-ODN (H) with either 1 (Fig 2, B) or 2 immunizations (data not shown), but differences were not statistically significant.

ODN conjugation masks allergen Ig-binding epitopes

Previous work from this laboratory suggested that ISS-ODN conjugation to allergens masked IgE binding.²¹ Consistent with these studies, Coomassie blue, which intercalates into protein hydrophobic regions, stained the OVA/ODN conjugates less effectively than native OVA (Fig 1). To confirm that ODN conjugation masked allergen-binding epitopes, we first conducted ELISA inhibition studies. As demonstrated in Fig 2, A, OVA/ISS-ODN (M) demonstrated significantly reduced binding to OVA-specific IgE and IgG relative to native OVA and OVA mixed with ISS-ODN. Furthermore, OVA/ISS-ODN (M) and OVA/M-ODN (M) demonstrated similar reductions in antibody binding relative to native OVA. Interestingly, we could not detect significant differences between low-, medium-, and high-ratio OVA/ISS-ODN conjugate binding to OVA hyperimmune

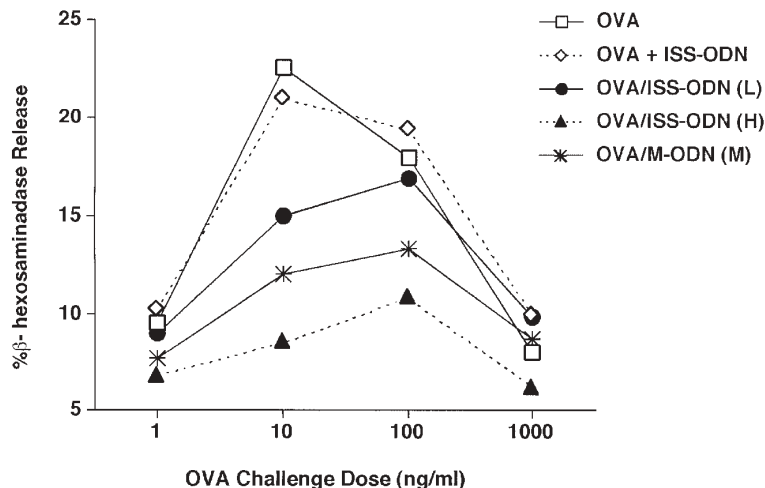


FIG 3. OVA/ODN conjugates are less allergenic than native OVA. BMMCs were sensitized with IgE-enriched OVA hyperimmune sera and then incubated with OVA, OVA plus ISS-ODN, or with one of the OVA/ODN conjugates over a range of concentrations (on the basis of OVA content), and BMMC degranulation was quantitated. Free ISS-ODN concentrations were based on content in OVA/ISS-ODN (H). Results represent mean β -hexosaminidase release for triplicate samples of each condition and are representative of 3 separate experiments ($P < .05$ for OVA vs OVA/ISS-ODN [L], OVA/ISS-M-ODN [M] vs OVA/ISS-ODN [L], and OVA/ISS-ODN [H] vs OVA/M-ODN [M]).

IgE or IgG in this assay system (data not shown).

Allergen engagement of IgE on the surface of mast cells plays a central role in the initial phase of many allergic reactions.²⁴ Therefore we next used flow cytometric techniques to compare the binding of FITC-labeled OVA, OVA/ISS-ODN (M), and OVA/M-ODN (M) to anti-OVA IgE-sensitized RBL cells. Sensitized RBL cells stained more brightly with OVA-FITC than unsensitized cells (Fig 2, B), indicating IgE-dependent binding. In contrast, anti-OVA IgE-dependent binding of FITC-labeled OVA/ISS-ODN (M) and OVA/M-ODN (M) could not be detected on RBL cells. By analogy, J774 cells incubated with anti-OVA sera enriched for IgG also stained brightly with OVA-FITC but only weakly with FITC-labeled OVA/ISS-ODN (M) or OVA/M-ODN (M; Fig 3, B).

OVA/ISS-ODN and OVA/M-ODN conjugates induce significantly less allergen-specific mast cell degranulation than native OVA

To determine whether ISS-ODN conjugation would effect OVA-dependent mast cell degranulation, BMMCs were sensitized with anti-OVA IgE incubated with OVA, OVA plus ISS-ODN, OVA/ISS-ODN(L or H), or OVA/M-ODN (M) over a range of concentrations, and β -hexosaminidase release was measured. AIC and OVA/M-ODN (M) induced significantly less β -hexosaminidase release than OVA (Fig 3). Furthermore, when OVA/ISS-ODN (L)–, OVA/M-ODN (M)–, and OVA/ISS-ODN (H)–induced BMMC degranulation were compared, a stepwise decrease in β -hexosaminidase release was seen

with each stepwise increase in ODN conjugation. Most impressively, nearly the same level of BMMC degranulation was seen with 100 ng of OVA/ISS-ODN (H) as with 1 ng of native OVA, whereas maximum β -hexosaminidase release by OVA/ISS-ODN (H) was reduced 3-fold compared with that by OVA. Finally, when compared with OVA alone, a mixture of OVA and ISS-ODN led to similar levels of BMMC degranulation.

OVA/ISS-ODN and OVA/M-ODN conjugates are less anaphylactogenic than native OVA

Because anaphylactic reactions are a life-threatening risk for allergic patients receiving immunotherapy, we determined whether AIC had less anaphylactogenic potential than native allergen. In this model challenges with native OVA and OVA plus ISS-ODN of OVA-sensitized mice led to 100% death (14/14 OVA-challenged and 10/10 OVA plus ISS-ODN–challenged mice; Table II). In contrast, an equivalent challenge with OVA/ISS-ODN (L), OVA/M-ODN (M), or OVA/ISS-ODN (H) led to 17%, 10%, or 0% death, respectively. Furthermore, postchallenge plasma histamine levels mirrored survival data, and OVA/ISS-ODN (H) challenge was shown to induce significantly less histamine release than OVA/ISS-ODN (L) or OVA/M-ODN (M) challenge.

OVA/ISS-ODN and OVA/M-ODN induce an attenuated Arthus reaction compared with native OVA

Although anaphylactic reactions are rare, other antibody-mediated reactions, such as large local reactions,

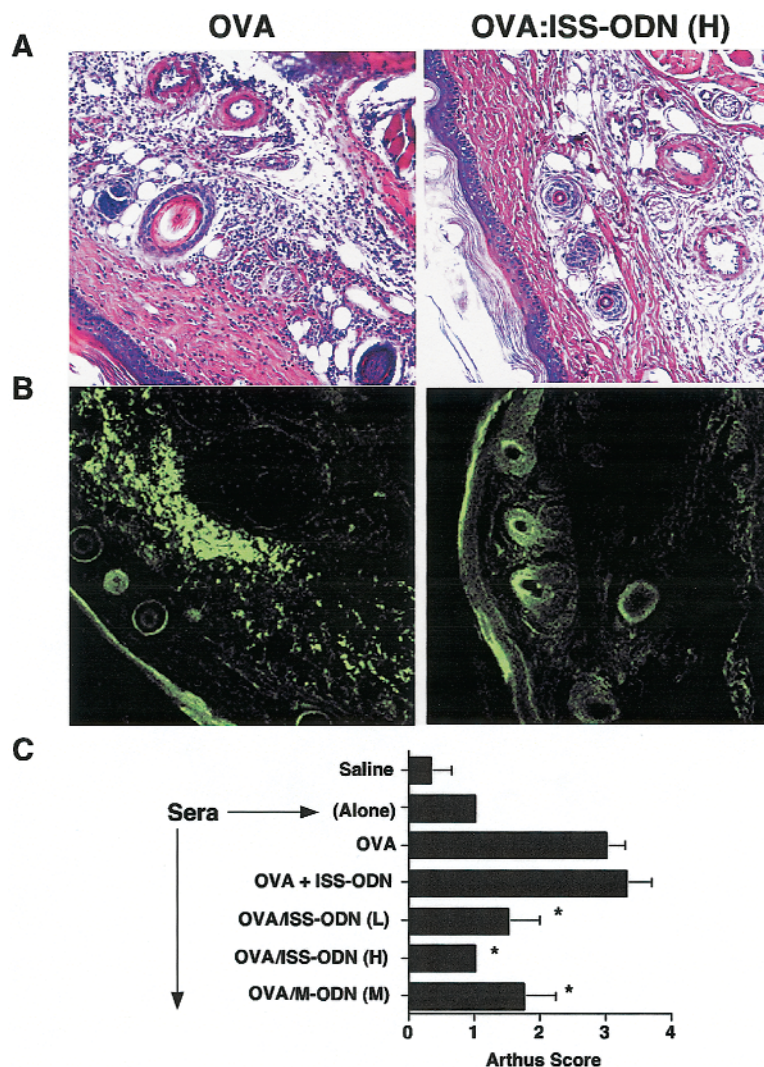


FIG 4. OVA/ODN conjugates induce weaker Arthus reactions than native OVA. Heat-inactivated OVA hyperimmune serum was mixed with OVA, OVA plus ISS-ODN, or one of the OVA/ODN conjugates. Free ISS-ODN was used at a dose corresponding to the content in OVA/ISS-ODN (H). These mixtures were intradermally injected into the tail base of naive mice. Six hours later, mice were killed, and tissue was harvested, fixed, sectioned, and stained with hematoxylin and eosin (**A**) or immunohistochemically stained for complement (C3) deposition (**B**). Sections were then scored for tissue inflammation and complement deposition (**C**). Results are presented as mean pathology scores $\pm$ SE ($n = 4$ mice/group; * $P < .05$ relative to OVA).

are common in patients receiving immunotherapy.^{2,4,6} Therefore we determined the potential of AIC, OVA/M-ODN (M), and OVA to induce a passive Arthus reaction. Equivalent amounts of OVA, OVA plus ISS-ODN, OVA/ISS-ODN (L or H), or OVA/M-ODN (M) were mixed with a constant amount of heat-inactivated anti-OVA hyperimmune sera, and the mixture was then intradermally injected into the base of the tails of naive mice. Tissue pathology from samples harvested 6 hours later was graded by a blinded observer. As with the anaphylaxis model, OVA/ISS-ODN (H) induced the weakest Arthus reaction relative to native OVA, whereas OVA/ISS-ODN (L) and OVA/M-ODN (M) induced intermediate reactions (Fig 4). As in the other allergenicity studies, free ISS-ODN did not inhibit the

development of the passive Arthus reaction when code-livered with OVA.

DISCUSSION

Previous attempts to improve the immunogenicity and reduce the allergenicity of immunotherapy reagents have failed.^{2,11,12} In these investigations we considered the immunogenic and allergenic consequences of chemically linking the immunostimulatory reagent ISS-ODN to OVA at different molar ratios. The results of these studies demonstrate that vaccination with OVA/ISS-ODN (L or H) leads to significantly stronger immune responses (Table I) than vaccination with OVA alone, OVA mixed with ISS-ODN, or OVA/M-ODN (Table II). Further-

more, OVA/ISS-ODN (L and H) and OVA/M-ODN (M) all demonstrated reduced allergenicity compared with native OVA and OVA mixed with ISS-ODN (Figs 3 and 4 and Table II). Taken together, the observations suggest that the principle of immunomodulatory reagent conjugation to allergens is a viable means to increase their immunogenicity and to reduce their allergenicity in general and anaphylactic potential in particular.

The observation that OVA/ISS-ODN vaccination stimulated significantly stronger IgG2a and cytokine responses than vaccination with OVA, OVA mixed with ISS-ODN, or OVA/M-ODN (M) strongly suggests that both the CpG motif and its conjugation to the target allergen contribute to the improved immunogenicity of AIC. However, although differences did not reach statistical significance, OVA/ISS-ODN (L) consistently induced a modestly higher immune response than OVA/ISS-ODN (H), with either 1 (Table I) or 2 immunizations (data not shown). Additional investigations are required to determine whether AIC formulations with ISS-ODN conjugation ratios of greater than 1:5.5 (OVA/ISS-ODN [H]) demonstrate further reductions in immunogenicity. It should be further noted that AIC vaccination induced a cytokine response dominated by IFN- γ and IL-10 because allergen-specific IFN- γ and IL-10 production have both been associated with successful allergen desensitization in clinical trials of immunotherapy.^{25,26}

The capacity of OVA/ISS-ODN to bind antibody from high-titer anti-OVA sera was also evaluated. OVA-specific IgE and IgG bound OVA/ISS-ODN (M) and OVA/M-ODN (M) significantly less efficiently than native OVA, both in terms of binding inhibition assays and by means of flow cytometry (Fig 2). Consistent with these binding assays, OVA/ISS-ODN (H and L) and OVA/M-ODN (M) induced significantly less degranulation from OVA IgE-sensitized BMMCs than native OVA, whereas OVA alone and OVA mixed with ISS-ODN induced similar levels of degranulation (Fig 3). Furthermore, a significant decrease in allergenicity was observed with each serial increase in the ODN/OVA conjugation ratio, and compared with OVA, 100-fold higher concentrations of OVA/ISS-ODN (H) were required to produce similar levels of degranulation from BMMCs. These results further demonstrated that although ODN conjugation was required for AIC's reduced in vitro allergenicity, the CpG motif that was critical for AIC's robust immunogenicity did not contribute to its reduced capacity for Ig binding or mast cell degranulation.

Although rare, anaphylaxis is a life-threatening risk for allergic patients receiving immunotherapy, whereas less severe antibody-mediated reactions, such as large local reactions at the injection site, are common.^{2,4,6} Therefore we determined the allergenicity of AIC in murine models of anaphylaxis and the Arthus reaction. In the anaphylaxis model challenge with OVA or OVA plus ISS-ODN led to 100% mortality, whereas OVA/ISS-ODN (L), OVA/M-ODN (M), and OVA/ISS-ODN (H) challenge led to only 17%, 10%, and 0% mortality, respectively. Furthermore, plasma histamine levels were

TABLE II. OVA/ODN conjugates are less anaphylactogenic than native OVA

Challenge	Histamine (μ M)	Survival (%)
Saline	ND	6/6 (100%)*
OVA	28 $\pm$ 2	0/14 (0%)
OVA + ISS-ODN	27 $\pm$ 1	0/10 (0%)
OVA/ISS-ODN (L)	15 $\pm$ 6†	10/12 (83%)*
OVA/ISS-ODN (H)	2 $\pm$ 1‡	10/10 (100%)*
OVA/M-ODN (M)	12 $\pm$ 1‡	9/10 (90%)*

Mice were sensitized to OVA and then intravenously challenged with OVA, OVA plus ISS-ODN, OVA/ISS-ODN (L or H), or OVA/M-ODN (M). All challenge doses were calculated on the basis of equivalence in OVA content, and free ISS-ODN was delivered at a dose corresponding to the ISS-ODN content of OVA/ISS-ODN (H). Histamine determinations (n = 7 mice/group) were conducted with plasma obtained 2 minutes after challenge, and results are presented as means $\pm$ SE. When lethal anaphylaxis occurred, it occurred within the first hour after challenge, and surviving mice looked well 24 hours later.

ND, None detected.

*P < .001 relative to OVA challenge.

†P < .05 relative to OVA challenge.

‡P < .05 compared with OVA/ISS-ODN (L) challenge.

significantly lower in OVA/ISS-ODN (H)-challenged mice compared with those seen in all other challenge groups (Table II). OVA and OVA mixed with ISS-ODN also induced more infiltration and complement deposition than OVA/ISS-ODN (L and H) and OVA/M-ODN (M) in a model of the Arthus reaction, and as with the anaphylaxis model, OVA/ISS (H) proved to be less allergenic than the other OVA/ODN conjugates (Fig 4). These in vivo studies of Ig-mediated hypersensitivity were consistent with the in vitro results in demonstrating that the allergenic potential of AIC is ODN conjugation dependent but not CpG motif dependent. The present results are also consistent with a recent phase I clinical trial, which demonstrated that AIC was less allergenic than allergen extract in end point titration skin testing studies of allergic patients.^{27,28}

Since Noon¹ described allergen immunotherapy in 1911, there have been few technologic or methodological advances in its use in clinical practice. However, the improved immunogenicity and decreased allergenicity of AIC relative to allergen alone, as described in these and other preclinical studies,^{14,20-22} suggests that it might well prove to be more effective and safe than traditional allergen extracts for the treatment of allergic diseases. Consistent with this premise, studies by Santeliz et al²⁹ and Shirota et al³⁰ have demonstrated the improved efficacy of AIC over allergen or allergen mixed with ISS-ODN alone in protecting allergic mice from asthma. In addition, several phase I clinical trials of AIC in patients with ragweed-induced allergic rhinitis have been completed with demonstrated safety and immunologic activity in small numbers of patients.²⁸ Our observations suggest that although higher levels of ISS-ODN conjugation might reduce the immunogenicity of AIC slightly, allergenicity and, in particular, anaphylactic potential are reduced significantly. Considered in the context of clinical applica-

tions, these findings indicate that for patients at highest risk for severe hypersensitivity reactions (ie, patients with anaphylactic hypersensitivity), high ISS-ODN conjugation ratio AIC would be safest for immunotherapy. However, if the ISS-ODN conjugation ratio is too high, there remains a theoretic concern that the immunogenicity of AIC might be compromised. The present investigations also suggest a rational approach for the standardization and optimization of AIC reagents for clinical use.

We thank Dr Robert Coffman for his critical review of this manuscript and Ms Nancy Noon and Ms Jane Uhle for their consistent-ly excellent secretarial assistance.

REFERENCES

- Noon L. Prophylactic inoculation against hay fever. *Lancet* 1911;1:1572-3.
- Creticos PS. Immunotherapy. In: Kaplan AP, editor. *Allergy*. 2nd ed. Philadelphia: Saunders; 1997. p. 726-39.
- Van Metre TE Jr, Adkinson NF Jr, Amodio FJ, et al. A comparison of immunotherapy schedules for injection treatment of ragweed pollen hay fever. *J Allergy Clin Immunol* 1982;69:181-93.
- Møllerup MT, Hahn GW, Poulsen LK, Malling H. Safety of allergen-specific immunotherapy. Relation between dosage regimen, allergen extract, disease and systemic side-effects during induction treatment. *Clin Exp Allergy* 2000;30:1423-9.
- Nelson HS, Lahr J, Rule R, Bock A, Leung D. Treatment of anaphylactic sensitivity to peanuts by immunotherapy with injections of aqueous peanut extract. *J Allergy Clin Immunol* 1997;99:744-51.
- Tankersley MS, Walker RL, Butler WK, Hagan LL, Napoli DC, Freeman TM. Safety and efficacy of an imported fire ant rash immunotherapy protocol with and without prophylactic treatment. *J Allergy Clin Immunol* 2002;109:556-62.
- Winther L, Malling HJ, Moseholm L, Mosbech H. Allergen-specific immunotherapy in birch- and grass-pollen-allergic rhinitis. I. Efficacy estimated by a model reducing the bias of annual differences in pollen counts. *Allergy* 2000;55:818-26.
- Adkinson NF Jr, Eggleston PA, Eney D, et al. A controlled trial of immunotherapy for asthma in allergic children. *N Engl J Med* 1997;336:324-31.
- Creticos PS, Reed CE, Norman PS, et al. Ragweed immunotherapy in adult asthma. *N Engl J Med* 1996;334:501-6.
- Creticos PS, Marsh DG, Proud D, et al. Responses to ragweed-pollen nasal challenge before and after immunotherapy. *J Allergy Clin Immunol* 1989;84:197-205.
- Norman PS, King TP, Alexander JF Jr, Kagey-Sobotka A, Lichtenstein LM. Immunologic responses to conjugates of antigen E in patients with ragweed hay fever. *J Allergy Clin Immunol* 1984;73:782-9.
- Norman PS, Lichtenstein LM, Marsh DG. Studies on allergoids from naturally occurring allergens. IV. Efficacy and safety of long-term allergoid treatment of ragweed hay fever. *J Allergy Clin Immunol* 1981;68:460-70.
- Horner AA, Widhopf GF, Burger JA, et al. Immunostimulatory DNA inhibits IL-4-dependent IgE synthesis by human B cells. *J Allergy Clin Immunol* 2001;108:417-23.
- Horner AA, Datta SK, Takabayashi K, et al. Immunostimulatory DNA-based vaccines elicit multifaceted immune responses against HIV at systemic and mucosa sites. *J Immunol* 2001;167:1584-91.
- Klinman DM, Yi AK, Beauchage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci U S A* 1996;93:2879-83.
- Hartmann G, Weiner GJ, Krieg AM. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc Natl Acad Sci U S A* 1999;96:9305-10.
- Sparwasser T, Koch ES, Vabulas RM, et al. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur J Immunol* 1998;28:2045-54.
- Horner AA, Van Uden JH, Zubeldia JM, Broide D, Raz E. DNA-based immunotherapeutics for the treatment of allergic disease. *Immunol Rev* 2001;179:102-18.
- Park Y, Chang YS, Lee SW, et al. The enhanced effect of a hexameric deoxyriboguanosine run conjugation to CpG oligodeoxynucleotides on protection against allergic asthma. *J Allergy Clin Immunol* 2001;108:570-6.
- Tighe H, Takabayashi K, Schwartz D, et al. Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. *Eur J Immunol* 2000;30:1939-47.
- Tighe H, Takabayashi K, Schwartz D, et al. Conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol* 2000;106:124-34.
- Shirota H, Sano K, Hirasawa N, et al. Novel roles of CpG oligodeoxynucleotides as a leader for the sampling and presentation of CpG-tagged antigen by dendritic cells. *J Immunol* 2001;167:66-74.
- Horner AA, Nguyen MD, Ronaghy A, Cinman N, Verbeek S, Raz E. DNA-based vaccination reduces the risk of lethal anaphylactic hypersensitivity in mice. *J Allergy Clin Immunol* 2000;106:349-56.
- Marquardt DL, Walker LL. Dependence of mast cell IgE-mediated cytokine production on nuclear factor-kappaB activity. *J Allergy Clin Immunol* 2000;105:500-5.
- Akdis CA, Blaser K. Role of IL-10 in allergen-specific immunotherapy and normal response to allergens. *Microbes Infect* 2001;3:891-8.
- Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Müller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. *J Immunol* 1995;154:4187-94.
- Creticos PS, Balcer SL, Schroeder JT, et al. Initial immunotherapy trial to explore the safety, tolerability, and immunogenicity of subcutaneous injection of the Amb a 1 immunostimulatory oligonucleotide conjugate (AIC) in ragweed allergic adults [abstract]. *J Allergy Clin Immunol* 2001;107(suppl):S216.
- Creticos PS, Eiden JJ, Balcer SL, et al. Immunostimulatory oligodeoxynucleotides conjugated to Amb a 1: safety, skin test reactivity, and basophil histamine release [abstract]. *J Allergy Clin Immunol* 2000;105(suppl):S70.
- Santeliz JV, Van Nest G, Traquina P, Larsen E, Wills-Karp M. Amb a 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. *J Allergy Clin Immunol* 2002;109:455-62.
- Shirota H, Sano K, Kikuchi T, Tamura G, Shirato K. Regulation of murine airway eosinophilia and Th2 cells by antigen-conjugated CpG oligodeoxynucleotides as a novel antigen-specific immunomodulator. *J Immunol* 2000;164:5575-82.