

Immunostimulatory DNA inhibits IL-4-dependent IgE synthesis by human B cells

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Background: Immunostimulatory sequence oligodeoxynucleotide (ISS-ODN) is a potent antiallergic immunomodulating agent in mice. However, few studies have addressed its antiallergic potential in human subjects.

Objective: We sought to determine whether a phosphorothioate ISS-ODN could inhibit IL-4-dependent IgE synthesis by human B cells.

Methods: Initially, nonatopic- and atopic-donor PBMCs were incubated with ISS-ODN or mutated oligodeoxynucleotide, and cytokine production and B-cell expression of IFN- γ receptor and IL-4 receptor were measured by using ELISA and flow cytometry, respectively. In subsequent studies atopic-donor PBMCs were incubated with IL-4 alone or with ISS-ODN or mutated oligodeoxynucleotide. After 14 days, IgE production and IgM, IgG, and IgA production were determined by using ELISA. In select IgE studies cytokines were neutralized with mAbs.

Results: ISS-ODN induced IL-12, IFN- α , IFN- γ , IL-10, and IL-6 production from both nonatopic- and atopic-donor PBMCs. ISS-ODN also increased IFN- γ receptor and inhibited IL-4 receptor expression on B cells from both donor populations. Furthermore, ISS-ODN inhibited IL-4-dependent IgE production by atopic-donor PBMCs. Neutralization of IL-12, IFN- α , IFN- γ , and IL-10, but not IL-6, attenuated the inhibitory activity of ISS-ODN on IgE production. In contrast to its inhibition of IgE synthesis, ISS-ODN stimulated the production of IgM, IgG, and IgA.

Conclusion: These in vitro studies demonstrate that phosphorothioate ISS-ODN elicits an innate immune response by PBMCs, which inhibits IL-4-dependent IgE synthesis. In addition, these results provide further support for consideration of ISS-ODN therapy for the treatment of allergic disease in clinical practice. (*J Allergy Clin Immunol* 2001;108:417-23.)

Key words: Human, B lymphocytes, allergy, isotype switching, vaccination, IL-4 receptor, IFN- γ receptor, CpG motif, immunostimulatory DNA

Abbreviations used

IFN- γ R: IFN- γ receptor

IL-4R: IL-4 receptor

ISS-ODN: Immunostimulatory sequence oligodeoxynucleotide

MFIR: Mean fluorescence intensity ratio

M-ODN: Mutated control oligodeoxynucleotide

Phosphorothioate immunostimulatory sequence oligodeoxynucleotide (ISS-ODN) has been shown to be a potent T_H1 and mucosal adjuvant and to effectively inhibit T_H2-biased immune deviation and the allergic phenotype in murine models of anaphylaxis, asthma, and conjunctivitis.¹⁻⁹ These ISS-ODN-induced effects on adaptive immunity are thought to principally be due to the production of type 1 cytokines (ie, IL-12, IFN- γ , and IFN- α), IL-6, and IL-10, as well as the expression of costimulatory molecules by ISS-ODN-activated antigen-presenting cells, B cells, and natural killer cells.^{2,3,10-12} However, although the immunostimulatory and antiallergic activities of phosphorothioate ISS-ODN are well established in mice, its effects on human immunity are only beginning to be studied.

It has been shown that phosphorothioate ISS-ODN activates human natural killer cells, B cells, macrophages, and dendritic cells to express costimulatory molecules (ie, CD40 and CD86) and to secrete cytokines (ie, IL-12, IL-18, IL-6, and IFN- γ).¹³⁻²⁰ It has also been demonstrated that phosphorothioate ISS-ODN induces IFN- γ and inhibits IL-4 production from mitogen-stimulated human T_H2 cell lines and leads to an approximately 33% reduction in spontaneous IgE release from human B cells.^{19,21} Recently, Fujieda et al²⁰ have further demonstrated that phosphodiester ISS-ODN can inhibit IgE synthesis by IL-4/anti-CD40-stimulated PBMCs by 50% to 75%. However, completed and ongoing phase 1 trials with allergic patients have used phosphorothioate ISS-ODN instead of phosphodiester ISS-ODN because of its resistance to DNase degradation and increased immunologic activity in mice.²²⁻²⁴

To further characterize the antiallergic potential of phosphorothioate ISS-ODN in human subjects, we conducted the following series of in vitro studies with PBMCs from both nonatopic and atopic donors. Consistent with previous investigations, phosphorothioate ISS-

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ODN induced IL-12, IFN- γ , and IL-6 production by PBMCs from both nonatopic and atopic donors.^{18,19} In addition, phosphorothioate ISS-ODN induced the production of IFN- α and IL-10 and increased nonatopic and atopic B-cell expression of CD119 (IFN- γ receptor [IFN- γ R]), while inhibiting CD124 (IL-4 receptor [IL-4R]) expression. Furthermore, phosphorothioate ISS-ODN almost completely inhibited IL-4-dependent IgE production of PBMCs from atopic donors ($\geq 95\%$ in most experiments), and cytokine neutralization studies identified IL-12, IFN- α , IFN- γ , and IL-10 as contributing to this activity. Finally, in contrast to their inhibition of IgE synthesis, phosphorothioate ISS-ODN stimulated the production of other immunoglobulin isotypes. Taken together, these in vitro findings suggest that phosphorothioate ISS-ODN may have both immunostimulatory and antiallergic activities in human subjects.

METHODS

Blood donors

Blood was obtained in accordance with the regulations of the human subjects committee at our institution. Laboratory coworkers and allergy clinic patients ($n = 9$) with a history of allergic rhinitis, asthma, or both and positive skin prick test reactions (wheal >3 mm larger than that produced by the saline control) to at least 1 allergen (Der p 1, Der f 1, tree mix, grass mix, weed mix, dog, and/or Fel d 1) served as a source of atopic-donor PBMCs. PBMCs were also derived from nonatopic donors ($n = 9$) with a negative history for allergic rhinitis and asthma symptoms and negative skin prick test reactions.

Oligodeoxynucleotides

CpG-containing phosphorothioate ISS-ODN and control phosphorothioate mutated oligodeoxynucleotide (M-ODN) were purchased from Trilink Biotechnologies (San Diego, Calif). The ISS-ODN used in these studies had the sequence 5'-TGACTGTGAACGTTGAGATGA-3', and the M-ODN had the sequence 5'-TGACTGTGAAGGT-TAGAGATGA-3'. These synthetic HPLC-purified phosphorothioate ODN preparations had no detectable endotoxin, as determined with a limulus amoebocyte lysate assay (Bio-Whittaker, Walkersville, Md).

Cytokine analysis

PBMCs were isolated by means of centrifugation over Ficoll (Pharmacia, Upsala, Sweden), washed, and resuspended in RPMI-1640 medium supplemented with 10% FCS, penicillin, streptomycin, and glutamine (Gibco BRL, Grand Island, NY; Bio-Whittaker; Irvine Scientific, Santa Ana, Calif; complete RPMI media) at a final concentration of 4×10^6 cells/mL. ISS-ODN and M-ODN were added to PBMC cultures at 10 μ g/mL, and supernatants were harvested after incubation for 48 hours at 37°C in 5% CO₂. IL-12 (Biosource, Camarillo, Calif), IFN- α , IFN- γ , IL-6, and IL-10 (Endogen, Woburn, Mass) levels were determined by using ELISA kits, according to the manufacturers' recommendations. Supernatants were compared with a standard curve of the appropriate cytokine on each plate by using the DeltaSOFT II version 3.66 program.

Flow cytometry

PBMCs were cultured with ISS-ODN, with M-ODN, or without ODN, as described above. Samples were stained with FITC-conjugated anti-CD19 and PE CD119 (IFN- γ R) or their isotype controls (PharMingen, San Diego, Calif) at saturating concentrations for 30 minutes at 4°C to measure IFN- γ R expression. For CD124 (IL-4R) staining, a signal amplification kit was used (Flowamp, Cleveland,

Ohio) to improve the sensitivity of the assay because this cytokine receptor is expressed on B cells at extremely low levels.²⁵ Briefly, cells were incubated with unconjugated anti-IL-4R (M57; Immunex, Seattle, Wash) or its isotype control mAb, followed by horseradish peroxidase-labeled anti-Ig and incubation with a substrate that is converted into a highly reactive biotin species in the presence of horseradish peroxidase. Biotin-labeled B cells were then detected with streptavidin-FITC, and CD19-PE was added during this incubation step to identify B cells. Analysis of cells was conducted with a FACSCalibur flow cytometry unit. Gating on CD19⁺ B cells, 5000 to 10,000 B-cell events were recorded per sample. The mean fluorescence intensity ratio (MFIR) was used to evaluate B-cell expression of cytokine receptors because it accounts for changes in B-cell autofluorescence and isotype control binding after ISS-ODN stimulation. The MFIR was calculated as follows:

$$\frac{(\text{Surface molecule specific mean fluorescence intensity})}{(\text{Isotype control mean fluorescence intensity})}$$

Flow cytometry data were analyzed with FlowJo 3.0.6 software (Tree Star, Inc, San Carlos, Calif).

Antibody production

PBMCs were cultured at a concentration of 1.5×10^6 cells/mL in complete RPMI media. IL-4 (5 ng/mL), ISS-ODN (10 μ g/mL), M-ODN (10 μ g/mL), and anti-CD40 mAb (1 μ g/mL, PharMingen) were added to selected cultures. In some experiments neutralizing antibodies for IL-12 (10 μ g/mL, PharMingen), IL-10 (10 μ g/mL, PharMingen), IFN- γ (10 μ g/mL, PharMingen), IFN- α (1 μ g/mL, Biosource), or IL-6 (10 μ g/mL, PharMingen) were added to cultures. Optimal concentrations of cytokine-neutralizing mAbs were determined by means of titration in preliminary experiments (data not shown). All supernatants were harvested at 14 days, and antibody levels were measured with ELISA. For IgE analysis, 96-well plates were coated with mouse anti-human IgE at 2 μ g/mL in 0.1 mol/L bicarbonate solution (hybridoma CIA-7.12; kindly provided by Dr Andrew Saxon, UCLA, Los Angeles, Calif). The plates were incubated overnight at 4°C and blocked with 0.1% gelatin solution, and then undiluted samples and IgE standards were added. The plates were then incubated at 4°C overnight and washed, and alkaline phosphatase-labeled goat anti-human IgE (Kirkegaard and Perry, Gaithersburg, Md) was added. The plates were washed after 2 hours, and *p*-nitrophenyl phosphate substrate buffer was then added. Absorbance was read 2 hours later at 405 to 650 nm. For IgM, IgG, and IgA ELISA, reagents were purchased from Kirkegaard and Perry, and for IgG subclass analysis, kits were purchased from the Binding Site (San Diego, Calif). These kits were used according to the manufacturer's directions. Samples were compared with the appropriate immunoglobulin isotype curve on each plate by using DeltaSOFT II v. 3.66 software.

Statistics

Statistical analyses were conducted with Statview software. Two-tailed paired Student *t* tests were conducted to analyze cytokine and antibody data. Flow cytometry data were analyzed with the Wilcoxon signed rank test.

RESULTS

ISS-ODN induces a similar cytokine response in PBMCs from both nonatopic and atopic donors

Freshly isolated PBMCs from nonatopic and atopic donors were incubated with ISS-ODN for 48 hours to determine the spectrum of cytokines produced by human

TABLE I. ISS-ODN-induced cytokine production from nonatopic- and atopic-donor PBMCs

	IL-12 (pg/mL)	IFN- α (pg/mL)	INF- γ (pg/mL)	IL-10 (pg/mL)	IL-6 (pg/mL)
Nonatopic					
Media	13 $\pm$ 8	>5	>5	66 $\pm$ 28	10 $\pm$ 5
ISS-ODN	180 $\pm$ 46*	240 $\pm$ 79*	119 $\pm$ 15*	644 $\pm$ 170*	240 $\pm$ 60*
M-ODN	64 $\pm$ 23	30 $\pm$ 13	21 $\pm$ 20	201 $\pm$ 39	36 $\pm$ 9
Atopic					
Media	25 $\pm$ 9	9 $\pm$ 5	>5	64 $\pm$ 43	7.5 $\pm$ 4
ISS-ODN	151 $\pm$ 19*	87 $\pm$ 18*	87 $\pm$ 20*	384 $\pm$ 60*	198 $\pm$ 48*
M-ODN	61 $\pm$ 5	8 $\pm$ 4	31 $\pm$ 15	181 $\pm$ 47	24 $\pm$ 8

PBMCs from 6 nonatopic and 6 atopic donors were cultured at 4×10^6 cells/mL in complete RPMI medium with ISS-ODN (10 μ g/mL), M-ODN (10 μ g/mL), or no ODN. Cultures were incubated for 48 hours, and supernatants were analyzed by means of ELISA. Cytokine results represent means $\pm$ SE for 4 donors. Statistically significant differences in cytokine production with ISS-ODN versus without ODN are identified with an asterisk.

* $P \leq .01$.

mononuclear cells in response to phosphorothioate ISS-ODN stimulation, and culture supernatants were analyzed by means of ELISA. As seen in Table I, both nonatopic- and atopic-donor PBMCs produced significant amounts of IL-12, IFN- α , IFN- γ , IL-10, and IL-6 on activation with ISS-ODN ($P \leq .01$). Although nonatopic-donor PBMCs generally produced more IFN- α and IL-10 than atopic-donor PBMCs, these differences did not reach statistical significance. Interestingly, both nonatopic- and atopic-donor PBMCs cultured with M-ODN produced increased amounts of cytokine when compared with PBMCs incubated without ODN. However, M-ODN induced consistently and significantly lower levels of each cytokine measured than ISS-ODN ($P \leq .05$).

ISS-ODN modulates the expression of IFN- γ R and IL-4R on B cells

PBMCs were cultured with ISS-ODN, stained for CD19 expression, and counterstained for expression of IFN- γ R or IL-4R to evaluate whether phosphorothioate ISS-ODN could modulate B-cell IFN- γ R and IL-4R expression. The MFIR was used to quantitate B-cell expression of IFN- γ R and IL-4R. Histograms of ISS-ODN-induced modulation of B-cell IFN- γ R and IL-4R expression from a representative atopic donor are presented in Fig 1, A, and in Fig 1, B, MFIR results from 5 nonatopic donors and 5 atopic donors are presented in scattergram format. B cells from the 2 donor populations demonstrated similar levels of IFN- γ R expression when left unstimulated, and ISS-ODN stimulation increased IFN- γ R expression on nonatopic and atopic B cells to a similar extent (35%-100%, $P = .043$). In contrast to IFN- γ R expression, ISS-ODN significantly reduced IL-4R expression on both nonatopic B cells and atopic B cells (15%-85%, $P = .043$). Interestingly, 3 of 5 atopic donors had B cells that expressed much higher levels of IL-4R without ISS stimulation than the rest of the atopic and nonatopic donors. Consistent with the cytokine studies, M-ODN also had a reproducible effect on cytokine receptor expression, but it was modest in comparison with that of ISS-ODN.

ISS-ODN inhibits IL-4-induced IgE production from atopic-donor PBMCs

Results reported above demonstrated that phosphorothioate ISS-ODN stimulated the release of antiallergic cytokines, such as IL-12, IFN- α , IFN- γ , and IL-10 from PBMCs, while increasing IFN- γ R expression and decreasing IL-4R expression on human B cells. These observations suggested that phosphorothioate ISS-ODN might also inhibit human B-cell responsiveness to IL-4. Atopic-donor PBMCs were cultured with IL-4 in the presence or absence of ISS-ODN for 14 days to test this hypothesis, and supernatant IgE levels were measured. As seen in Fig 2, PBMCs from 4 allergic donors produced significant amounts of IgE in response to culture with IL-4, whereas addition of ISS-ODN to PBMC/IL-4 cultures reduced IgE synthesis by approximately 95% ($P = .0016$ vs PBMC/IL-4 culture without ODN); IgE levels were often below the limits of detection for the assay system (400 pg/mL). Because B-cell ϵ isotype switching and IgE production require both IL-4 and a source of B-cell CD40 engagement, we also conducted experiments in which CD40 mAb was added to PBMCs cultured with IL-4 and ISS-ODN. However, CD40 stimulation did not rescue IL-4-stimulated PBMCs from the inhibitory effects of ISS-ODN, and IgE levels were again only 5% to 10% of the levels seen in control cultures (data not shown). As with other experiments, M-ODN also inhibited IgE synthesis from PBMCs cultured with IL-4, but its inhibitory activity did not reach statistical significance.

We next determined the role of cytokines in mediating the inhibitory activity of ISS-ODN on IL-4-dependent IgE production. PBMCs were cultured with IL-4 and ISS-ODN in the presence of optimized concentrations of cytokine-neutralizing mAbs. As presented in Fig 3, in experiments with 3 additional atopic donors, neutralization of IL-12 ($P = .023$), IFN- α ($P = .002$), IFN- γ ($P = .044$), and IL-10 ($P = .048$), but not IL-6, attenuated the inhibitory effects of ISS-ODN on IL-4-mediated IgE production.

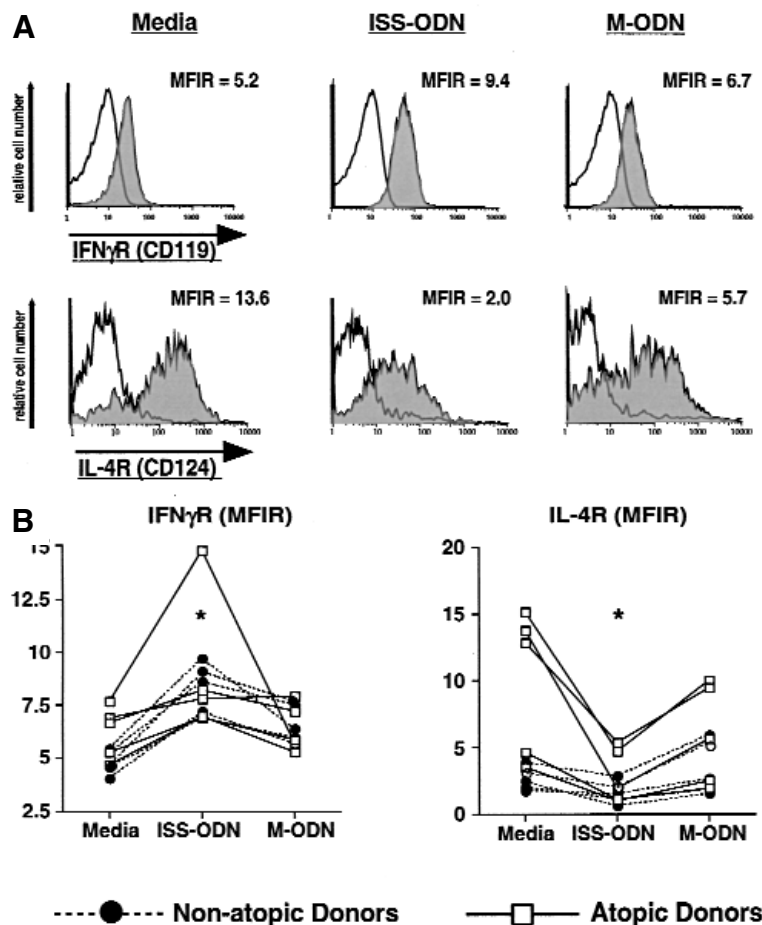


FIG 1. Effect of ISS-ODN on atopic- and nonatopic-donor B-cell expression of IFN- γ R and IL-4R. PBMCs from atopic and nonatopic donors were cultured as described in Table I and the "Methods" section. After 48 hours, IFN- γ R α (CD119) and IL-4R α (CD124) expression on CD19 $^{+}$ B cells was determined by using flow cytometry. The MFIR was used to quantitate IFN- γ R and IL-4R expression. **A**, A representative atopic donor is presented, with cytokine receptor expression in gray and isotype control expression in white. **B**, Results of ISS-ODN-induced changes in the MFIR for IFN- γ R and IL-4R expression from a total of 5 nonatopic and 5 atopic donors are represented in scattergraph format. Statistically significant differences in cytokine receptor expression with ISS-ODN stimulation versus without ODN stimulation were found. * $P = .043$ for both nonatopic- and atopic-donor B cells.

ISS-ODN stimulates IgM, IgG, and IgA synthesis from PBMCs cultured with IL-4

Supernatants from human PBMC/IL-4/ISS-ODN cultures used to determine IgE responses ($n = 7$) were additionally analyzed for IgM, IgG, and IgA content to evaluate the effect of ISS-ODN on B-cell production of immunoglobulins other than IgE. As shown in Fig 4, compared with atopic-donor PBMCs cultured with IL-4 alone, coculture with IL-4 and ISS-ODN led to a 15-fold increase in IgM ($P = .0003$), a 4-fold increase in IgG ($P = .0012$), and a 2-fold increase in IgA ($P = .027$) production. M-ODN also modestly increased IgM, IgG, and IgA synthesis by PBMCs cultured with IL-4, but these increases were not statistically significant. ISS-ODN-induced IgM, IgG, and IgA production was not dependent on the addition of exogenous IL-4 because

PBMC cultures with ISS-ODN alone, as well as those with ISS-ODN and IL-4, had similar levels of IgM, IgG, and IgA in their supernatants. Because total IgG production was increased by addition of ISS-ODN to PBMC/IL-4 cultures, IgG subclass synthesis was analyzed further. These studies demonstrated that addition of ISS-ODN to PBMC/IL-4 cultures led to symmetric increases in the production of all IgG subclasses (data not shown).

DISCUSSION

The present series of experiments were designed to evaluate the immunostimulatory and antiallergic activities of phosphorothioate ISS-ODN on nonatopic- and atopic-donor PBMCs. These investigations demonstrated that phosphorothioate ISS-ODN induces the synthesis of IL-12, IFN- α , IFN- γ , IL-10, and IL-6 by both nonatopic-

and atopic-donor PBMCs and increases IFN- γ R expression and decreases IL-4R expression on B cells derived from both donor groups. In addition, ISS-ODN was shown to inhibit IL-4-dependent IgE synthesis by atopic-donor PBMCs, and cytokine-neutralization experiments identified IL-12, IFN- α , IFN- γ , and IL-10, but not IL-6, as contributing to the inhibitory influence of ISS-ODN on IgE production. In contrast to its inhibition of IL-4-dependent IgE synthesis, ISS-ODN was further shown to stimulate the production of IgM, IgG, and IgA, both in the presence and absence of exogenous IL-4.

A previous study reported that phosphorothioate ISS-ODN modestly inhibits spontaneous IgE synthesis (33% decrease) from IgE-precommitted human B cells,¹⁹ and Fujieda et al²⁰ have gone on to show that phosphodiester ISS-ODN inhibits IgE synthesis from PBMCs cultured with IL-4 and CD40. Our finding that ISS-ODN inhibits IL-4-dependent human IgE synthesis is consistent with these results. However, in consideration of the studies of Fujieda et al, it should be noted that phosphodiester ISS-ODN were generally used at a 50 μ g/mL (5 μ mol/L) concentration, and in the majority of their experiments, IL-4/CD40-mediated IgE production was inhibited by 50% to 75%. The present studies used phosphorothioate ISS-ODN at a 5-fold lower concentration (10 μ g/mL) and found that IgE synthesis by both IL-4- and IL-4/CD40-stimulated PBMCs was inhibited by approximately 95%. The improved efficacy of phosphorothioate ISS-ODN in this human IgE synthesis model is not completely surprising because the phosphorothioate backbone is relatively resistant to DNase digestion, and phosphorothioate ISS-ODN is more potent than phosphodiester ISS-ODN in mice.²⁴ However, the observation is important because phosphorothioate ISS-ODN has already been used to treat allergic patients in clinical trials.^{22,23}

Many of the cytokines (ie, IL-12, IFN- α , IFN- γ , and IL-10) produced in response to ISS-ODN have been shown to inhibit IgE synthesis and other IL-4-dependent responses.²⁶⁻³¹ Therefore we pursued the mechanisms of ISS-ODN-mediated inhibition of IgE synthesis with cytokine-neutralization experiments. Consistent with a previous study, ISS-ODN-induced IL-12 and IFN- γ production was shown to contribute to its inhibition of IgE synthesis.²⁰ However, the present results further establish that IFN- α and IL-10 also mediate the inhibitory activity of ISS-ODN on human IgE synthesis. In addition, these investigations suggest that ISS-ODN-induced upregulation of IFN- γ R and downregulation of IL-4R could also contribute to its reported antiallergic activities in mice and in vitro human systems.^{2,4-6,9,19-21} Although we are unaware of studies suggesting that regulation of IFN- γ R and IL-4R expression contributes to the T_H bias of adaptive immune responses, IL-4R polymorphisms associated with a gain in function have been genetically linked to the allergic phenotype.³²⁻³⁴ In this vein unstimulated B cells from several atopic donors used in these investigations had 2- to 3-fold higher levels of IL-4R expression when compared with B cells from the rest of the atopic and nonatopic donors. The role of ISS-ODN-mediated

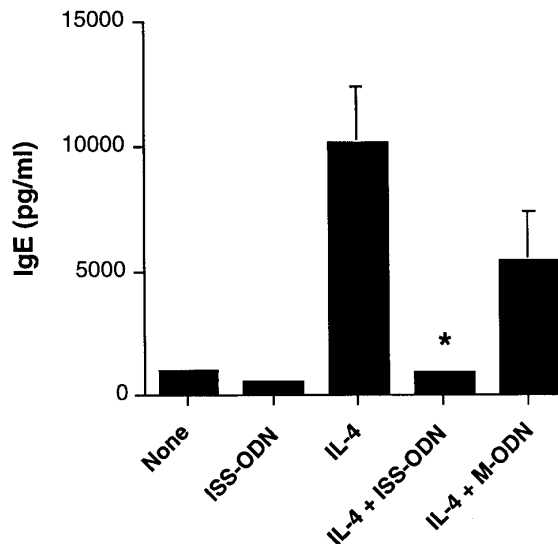


FIG 2. Effect of ISS-ODN on IL-4-induced IgE production by PBMCs. PBMCs were cultured at 1.5×10^6 cells/mL in complete RPMI with IL-4 (5 ng/mL), with IL-4 plus ISS-ODN (10 μ g/mL) or M-ODN (10 μ g/mL), or without additions. After 14 days, culture supernatants were harvested and analyzed by means of ELISA for IgE content. Results represent mean IgE production $\pm$ SE by PBMCs from 4 atopic patients. PBMCs cultured with IL-4 and ISS-ODN produced significantly less IgE than PBMCs cultured with IL-4 alone. * $P = .0016$.

cytokine production and cytokine receptor expression on the relative responsiveness of B cells to IL-4 requires further investigation. However, results presented herein suggest that ISS-ODN inhibits human IgE synthesis by means of redundant and complementary mechanisms and provide a potential explanation for the attenuated but continued antiallergic activity of ISS-ODN in IL-12 and IFN- γ single knock-out and IL-12/IFN- γ double knock-out mice.³⁵

Consistent with previous studies, these investigations demonstrate that phosphorothioate ISS-ODN promotes IgM, IgA, and IgG production from human B cells.^{16,24} ISS-ODN was also shown to increase IFN- γ R expression and decrease IL-4R expression and to inhibit IL-4-dependent IgE synthesis. When considered together, these observations suggest that ISS-ODN specifically inhibits IL-4 responsiveness, while promoting other B-cell activities.

Although clearly less effective than phosphorothioate ISS-ODN, the phosphorothioate M-ODN used in the present investigations also elicited PBMC responses. Interestingly, in murine studies the same M-ODN has no detectable immunologic activity (unpublished data). Other investigators have published similar findings and have suggested that human, unlike murine, mononuclear cells are susceptible to sequence-independent stimulation by phosphorothioate oligonucleotides.^{16,21} Alternatively, there may be as-yet-unidentified DNA sequences that have immunostimulatory properties in human subjects. It has already been shown that a number of ODNs containing CpG dinucleotides without

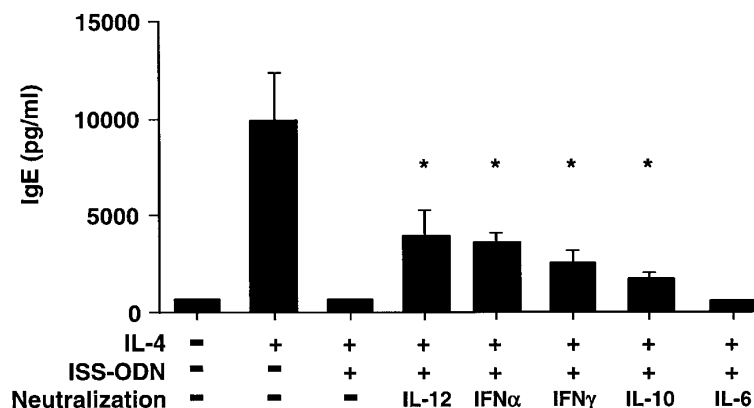


FIG 3. Effects of IL-12, IFN- α , IFN- γ , IL-10, and IL-6 neutralization on ISS-ODN-mediated inhibition of IL-4-dependent IgE synthesis. PBMCs were cultured with IL-4 alone or with IL-4 plus ISS-ODN as described in Fig 2 and the "Methods" section. Cytokine-neutralizing mAbs were added to selected cultures. After 14 days, culture supernatants were harvested for IgE determination. Results represent mean IgE production $\pm$ SE by PBMCs from 3 atopic donors. Statistically significant differences in IgE production by PBMCs cultured with IL-4/ISS versus IL-4/ISS plus a cytokine-neutralizing mAb are identified with an asterisk. * $P \leq .05$.

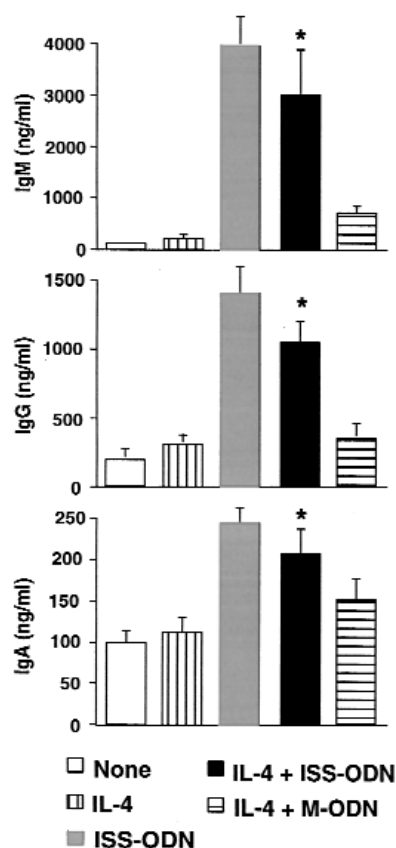


FIG 4. Effect of ISS-ODN on IgM, IgG, and IgA production by PBMCs cultured with IL-4. PBMCs were cultured as described in Fig 2 and the "Methods" section. After 14 days, culture supernatants were harvested and analyzed by means of ELISA for IgM, IgG, and IgA content. Results represent the mean IgM, IgG, or IgA levels $\pm$ SE for the 7 atopic donors used in the studies presented in Figs 3 and 4. Statistically significant differences in immunoglobulin production by PBMCs cultured with IL-4 and ISS-ODN versus PBMCs cultured without ODN were found. * $P \leq .05$.

the flanking sequences that define the ISS-ODN motif in mice have activity in human systems.^{16,21} Furthermore, the observations of Hartmann et al³⁶ suggest that the immunostimulatory DNA sequences, which optimally activate human and murine mononuclear cells, differ. Considering the outbred nature of the human population and its consequent polymorphisms, it is conceivable that the optimal immunostimulatory DNA sequence might also differ for different individuals. Although this issue requires further investigation, the present series of experiments used 18 separate donors, both atopic and nonatopic, and all PBMC preparations demonstrated significant phosphorothioate ISS-ODN responsiveness.

In summary, the results presented in this article demonstrate that phosphorothioate ISS-ODN induces a similar response by mononuclear cells derived from nonatopic and atopic donors. This response includes (1) the production of IL-12, IFN- α , IFN- γ , IL-10, and IL-6 from PBMCs; (2) the upregulation of IFN- γ R and downregulation of IL-4R expression on B cells; (3) the inhibition of IL-4-dependent IgE production; and (4) the synthesis of IgM, IgG, and IgA. These findings are consistent with and extend the previously described activities of ISS-ODN. In addition, the results demonstrate that ISS-ODN inhibits human B-cell responsiveness to IL-4, in part by its stimulation of type 1 cytokines and IL-10 production, and suggest that its modulation of B-cell IFN- γ R and IL-4R expression further favors responsiveness to IFN- γ over IL-4. Although the in vitro systems used in this series of experiments do not equate with the treatment of allergic hypersensitivity states, they do allow a limited assessment of the activity of phosphorothioate ISS-ODN and its potential antiallergic activities in human subjects. Accepting the limitations of this system, these results are encouraging, and provide further support for ongoing evaluation of phosphorothioate ISS-ODN in clinical trials to assess its safety and efficacy in the treatment of allergic diseases.

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