

Synthetic Oligodeoxynucleotides Inhibit IgE Induction in Human Lymphocytes

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Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs have the capacity to stimulate T-helper (Th)1-type responses in mice. Th1 cytokines are known to act as downregulators of IgE production. In this study we investigated whether synthetic ODNs inhibited IgE production in human peripheral blood mononuclear cells (PBMC) from normal donors stimulated with interleukin (IL)-4 plus anti-CD40 monoclonal antibody (mAb) *in vitro*. Thirty-mer single-stranded ODNs were randomly selected from the complementary DNA encoding the MPB-70 of *Mycobacterium bovis* Bacillus Calmette-Guerin. Two ODNs, containing CGTACG or AACGTT inhibited IgE production by human PBMC. When other oligonucleotides were substituted in a portion of the sequence of the core or flanking oligonucleotides in the ODN containing CGTACG, ODNs containing NACGTCG or A/CTCGTCG sequences specifically inhibited IgE production by human PBMC *in vitro*. The inhibition of IgE production by certain ODNs was mediated by both interferon (IFN)- γ and IL-12, since the ODN-induced suppression was blocked by the addition of anti-IFN- γ or anti-IL-12 mAb. Also, the ODNs inhibited induction of ϵ germline transcripts by IL-4. Our findings indicate that synthetic ODNs appear to be candidates for the treatment of IgE-dependent allergic disease in humans.

During the past decade, the molecular and cellular mechanisms of regulation of IgE production have been substantially solved. In general, human B cells have been shown to produce IgE after stimulation with T-helper (Th)-2 cytokine (particularly interleukin [IL]-4) in the presence of CD40 ligand (1, 2), whereas T helper (Th)1 cytokines (interferon [IFN]- γ and interleukin [IL]-12) downregulate IgE production (1-3).

Bacterial DNA, but not vertebrate DNA, causes activation of B cells and natural killer (NK) cells, and the secretion of Th1 cytokines (4-7). These effects result from the presence of unmethylated CpG dinucleotide motifs. The exhaustive research of Krieg and colleagues showed that certain synthetic oligodeoxynucleotides containing an unmethylated CpG motif (CpG DNA) have the same immunostimulatory properties in mice as does bacterial DNA (5, 6, 8-11). Thus, CpG DNA also induces the production of IFN- γ , IL-12, IL-6, and tumor necrosis factor (TNF)- α in mice *in vivo* and in mouse NK or T cells *in vitro*.

These results have made DNA vaccination a promising candidate method for regulating allergic reactions and as an effective strategy for treating allergic disease. One major unit in gene vaccination done with plasmid DNA is a plasmid backbone that delivers adjuvant and mitogenic activity via immun-

ostimulatory sequences. Yamamoto and colleagues demonstrated for the first time that MY-1, a DNA-rich fraction extracted and purified from *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG), activated NK cells and enhanced IFN- γ production in mice (4). Additionally, they determined several sequences of 30-mer oligonucleotides that most potentially augmented the secretion of IFN- γ and NK activity (12-14). Most of their oligonucleotides included a CpG motif within a palindromic hexamer as an immunologically active sequence (12-14). Furthermore, Raz's group demonstrated that intradermal injection of plasmid DNA containing short immunostimulatory DNA sequences decreased antigen-specific IgE antibody production and induced Th1 immune responses in mice, and that the transfection of human peripheral blood mononuclear cells (PBMC) with oligonucleotides containing immunostimulatory DNA sequences resulted in the production of IFN- γ , IFN- α , IFN- β , and IL-12 by transfected cells (15-17).

In all of the studies that have been done of IgE production or that have used hypersensitivity models for asthma, mice have been used as the subjects. In the present study, we investigated whether CpG DNA inhibits IgE production by human PBMC stimulated with IL-4 and monoclonal anti-CD40 antibody *in vitro*. Because we had previously shown that MY-1 inhibited IgE production *in vitro* by PBMC stimulated with IL-4 plus anti-CD40 monoclonal antibody (mAb) (18), we designed the sequences of the synthetic oligodeoxynucleotides (ODNs) used in the present study on the basis of the sequence of MY-1 (13).

METHODS

Reagents

Mouse antihuman IgE mAbs (CIA-E-7.12 and CIA-E-4.15) were a gift from Dr. A. Saxon of the University of California, Los Angeles, CA. Anti-IL-12 receptor mAb (12R β .44) was a gift from Dr. J. Ritz of the Dana-Farber Cancer Institute, Boston, MA. Human IL-4 was the gift of Ono Pharmaceutical Co., Ltd. (Osaka, Japan), and anti-CD40 mAb G28-5 was a gift from Dr. E. A. Clark of the University of Washington, Seattle, WA. The following reagents were obtained commercially: mouse antihuman IgA mAb (Calbiochem, San Diego, CA), mouse antihuman IgG₁ mAb (Chemicon International, Inc., Temecula, CA), mouse anti-human IgG₄ mAb (Cosmobio, Tokyo, Japan), alkaline phosphatase-labeled goat antihuman IgE (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), alkaline phosphatase-labeled goat antihuman IgA (Biosource International, Camarillo, CA), alkaline phosphatase-labeled goat antihuman IgG (Biosource International), mouse antihuman IFN- γ mAb (Biosource International), mouse antihuman IL-12 mAb (Genzyme, Cambridge, MA), human IFN- γ enzyme-linked immunosorbent assay (ELISA) kit (Biosource), and ODNs (Nishinbo, Tokyo, Japan).

Cells and Cell Cultures

PBMC were isolated from 12 healthy volunteers by Ficoll-Hypaque centrifugation. PBMC were also isolated from three atopic patients with high total IgE titers in their serum. PBMC (1×10^6 cells/ml) were cultured for 14 d in complete medium prepared from RPMI supple-

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mented with 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin in the presence of IL-4 (100 U/ml) and anti-CD40 mAb (0.1 µg/ml), which represented optimal conditions for IgE production (2, 19). Each ODN (5 µM) was added simultaneously with IL-4 and anti-CD40. Anti-IFN-γ mAb (1 µg/ml) or anti-IL-12 mAb (20 µg/ml) was added to the culture for a blocking test.

CD56⁺ cells were isolated by using CD56 microbeads with a magnetic cell sorting system (MACS; Miltenyi Biotec, GmbH, Bergisch, Germany). The presence of less than 1% CD56⁺ cells in the fraction containing CD56⁻ cells was confirmed with flow cytometry.

Ig Measurements

Total IgE, IgA, IgG₁, and IgG₄ levels in the supernatants of stimulated cells were measured with ELISAs as previously described (2, 19). Briefly, microtiter plates were coated overnight at 4° C with two types of mAb to antihuman IgE (CIA-E-7.12 and CIA-E-4.15), or with a mAb to antihuman IgA, IgG₁, or IgG₄. After the wells were blocked with 0.1% gelatin for at least 1 h, 100-µl aliquots of culture supernatants were placed in duplicate wells, and the plates were incubated for 2 h at room temperature. After the plates were washed, alkaline phosphatase-labeled IgE, IgA, or IgG was added for antibody detection. Absorbance at 405 nm was read with an ELISA reader (Bio-Tek Instruments, Inc., Burlington, CA). The sensitivity of the assay for the IgE, IgA, IgG₁, and IgG₄ subclasses was 0.1 ng/ml.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction Strategy

Total messenger RNA (mRNA) was obtained from stimulated and nonstimulated human PBMC, using Trizol reagent (Life Technologies Inc., Tokyo, Japan), as previously described (20). Trizol is a monophasic solution of phenol and guanidine isothiocyanate that improves extraction over that with the single-step RNA isolation method. RNA suspended in 0.1% diethylpyrocarbonate-treated water was digested with deoxyribonuclease (DNase) I (Sigma, St. Louis, MO) to remove contaminating DNA, and was then extracted with phenol/chloroform and precipitated with ethanol. Total RNA (0.5 µg) was reverse-transcribed to complementary DNA (cDNA), using oligodeoxythymidine₁₅ (Boehringer-Mannheim Co., Indianapolis, IN) as primer and mouse Moloney leukemia virus reverse transcriptase (GIBCO BRL), under conditions recommended by the manufacturer.

All polymerase chain reaction (PCR) assays were run in 25-µl reactions containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 5% dimethylsulfoxide, 50 pM primer per reaction, and 2.5 U of *Taq* polymerase. For detection of ϵ germline transcripts and β actin, PCR was conducted for 30 cycles of 94° C for 1 min, 65° C for 1 min and 72° C for 1 min. The primer sequences for ϵ germline transcripts I ϵ were: AGGCTCCACTGCCCGGCACAGAAATA and C ϵ GGACAAGTCCACGTCCATGA; and for β actin were: TCACCAACTGGGACGACATGGAG and CTCCTTAATGTCACGCA-CGATTTC (20).

Detection of IFN-γ

The concentration of IFN-γ in the supernatants of PBMC stimulated with IL-4 and anti-CD40 mAb in the absence or in the presence of ODNs was measured with an ELISA kit. Protocols provided with the kit allow IFN-γ to be measured in the range of 4 to 1,000 pg/ml.

Statistics

Data are expressed as mean $\pm$ SEM. The statistical significance of effects on Ig production was determined with Wilcoxon's signed-ranks test.

RESULTS

Characterization of ODN Sequences Needed for Inhibition of IgE Production by PBMC

Previous studies demonstrated that a 30-mer ODN (5'-accgat-GACGTCgccggtgacggcaccacg-3') from MY-1, including one palindromic sequence, GACGTC, had the strongest activity in inducing IFN-γ production in mice (13, 14). Since it was found that a palindromic sequence (6-mer) was critical for the bio-

logic activity of this ODN, the effect of replacing GACGTC with each of the 63 theoretically possible six-base palindromic sequences was tested (13). When this work was done, 10 kinds of palindromic sequences were shown to have strong activity in inducing IFN-γ production by mouse spleen cells. In the present study, PBMC were cultured for 14 d with IL-4 plus anti-CD40 mAb in the presence of 10 ODNs (5 µM) active for the production of IFN-γ and one inactive ODN (ODN 1; 5 µM), and induction of IgE in the supernatant was measured with an ELISA. As shown in Table 1, two ODNs (ODN 2, containing an AACGTT palindrome, and ODN 4, containing a CG-TACG palindrome) inhibited the production of IgE induced by IL-4 plus anti-CD40 mAb in nine independent experiments, whereas the other nine ODNs had no effect on IgE production. None of the ODNs had any effect on the viability of PBMC stimulated with IL-4 plus anti-CD40 mAb, as shown by trypan blue dye exclusion (data not shown). Since ODN 4 most potently suppressed IgE production, all subsequent experiments were done with ODN 4.

Because Yamamoto and colleagues had stressed the importance of the palindromic sequence in the induction of IFN-γ (12–14), we investigated whether a palindromic sequence was crucial for the inhibition of IgE synthesis by human PBMC. The fourth base (adenine) in the palindromic sequence of ODN 4 (CGTACG) was replaced with three other bases. As shown in Figure 1, ODN 4 containing the palindromic sequence CG-TACG inhibited the production of IgE induced by IL-4 plus anti-CD40 mAb in a dose-dependent manner. ODN 14, which contains the core sequence, also inhibited IgE synthesis, but ODN 15, containing the sequence CGTGCG, and ODN 16, containing the sequence CGTCCG, did not. The maximal effect of ODN 14 in two experiments was obtained at 5 µM, which is consistent with the optimal CpG DNA concentration for activation of mouse lymphocytes (5–11). We therefore performed all subsequent experiments at an ODN concentration of 5 µM. In nine independent experiments done with PBMC from different donors, the suppression rate of IgE synthesis by ODN 14 was higher than that of ODN 4, suggesting that a palindromic sequence is not necessary for the inhibition of IgE synthesis by

TABLE 1
IMMUNOGLOBULIN E PRODUCTION BY HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS IN THE PRESENCE OF OLIGODEOXYNUCLEOTIDES CONTAINING 6-BASE PALINDROMES

ODN		
No.	Sequence (5'-3')	IgE (ng/ml)
(-)	(-)	2,500 $\pm$ 1,077
1	accgat ACCGGT gccggtgacggcaccacg	3,246 $\pm$ 1,233
2	 AACGTT	1,157 $\pm$ 469*
3	 GCGGCG	2,239 $\pm$ 949
4	 CGTACG	702 $\pm$ 238†
5	 CGGCCG	2,762 $\pm$ 1,107
6	 TCGCGA	2,920 $\pm$ 1,173
7	 GACGTC	3,253 $\pm$ 1,156
8	 AGCGCT	3,141 $\pm$ 1,084
9	 ATCGAT	2,671 $\pm$ 905
10	 CGATCG	2,667 $\pm$ 1,053
17	 CGCGCG	2,686 $\pm$ 1,183

Definition of abbreviations: mAb = monoclonal antibody; ODN = oligodeoxynucleotide; PBMC = peripheral blood mononuclear cell.

Human PBMC from healthy donors (n = 9) were cultured with IL-4 (100 U/ml) plus anti-CD40 mAb (0.1 µg/ml) in the presence of 5 µM of ODN. After 14 d of culture, the supernatants were harvested and the IgE concentration was measured. The results are expressed as mean $\pm$ SEM.

* p < 0.05 versus value in the absence of ODN.

† p < 0.01 versus value in the absence of ODN.

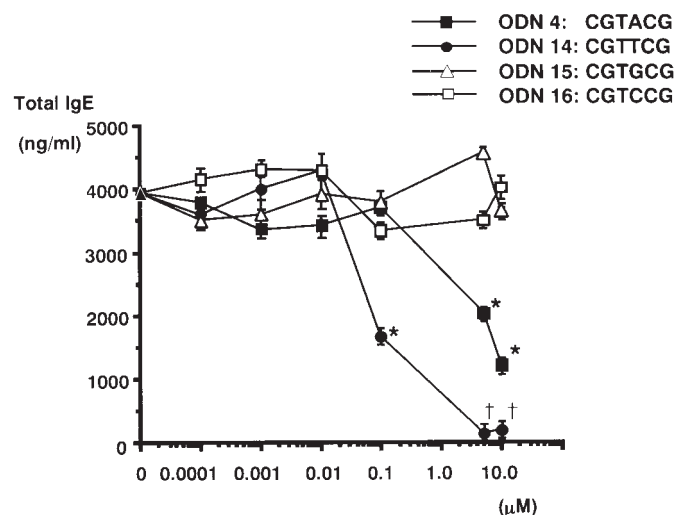


Figure 1. Effect of ODNs on IgE production. Human PBMC from non-atopic healthy donors were cultured with IL-4 (100 U/ml) and anti-CD40 mAb (0.1 μg/ml) for 14 d in the presence of various concentrations of ODNs. ODN 4 or ODN 14 inhibited IgE production in a dose-dependent manner. Representative data of two experiments are expressed as the mean $\pm$ SEM of triplicate samples. * p < 0.05, † p < 0.01 compared with IgE values for PBMC stimulated with IL-4 plus anti-CD40 mAb in the absence of ODN.

ODN (Table 2). Although a wide distribution of IgE production was observed in nine healthy volunteers, changes in IgE production by four ODNs, shown in Figure 2, indicated significant differences in IgE production after addition of the four different ODNs. In all cases, the addition of ODN 14 suppressed IgE production induced by stimulation with IL-4 and anti-CD40 mAb as compared with the control (p < 0.01). ODN 4 inhibited IgE production in eight of nine cases (p < 0.05).

To rule out a toxic effect of the ODN (5 μM) used in the study, apoptotic cells were stained according to standard procedure with fluorescein isothiocyanate-conjugated annexin V, and were examined flow cytometrically. The addition of four ODNs (ODN 4, ODN 14, ODN 15, ODN 16) to the PBMC culture after stimulation with IL-4 and anti-CD40 mAb did not influence the percentage of annexin V-positive cells as compared with that in the control, suggesting that the ODN (5 μM) was not toxic to PBMC.

This result led us to replace the third and fourth bases of

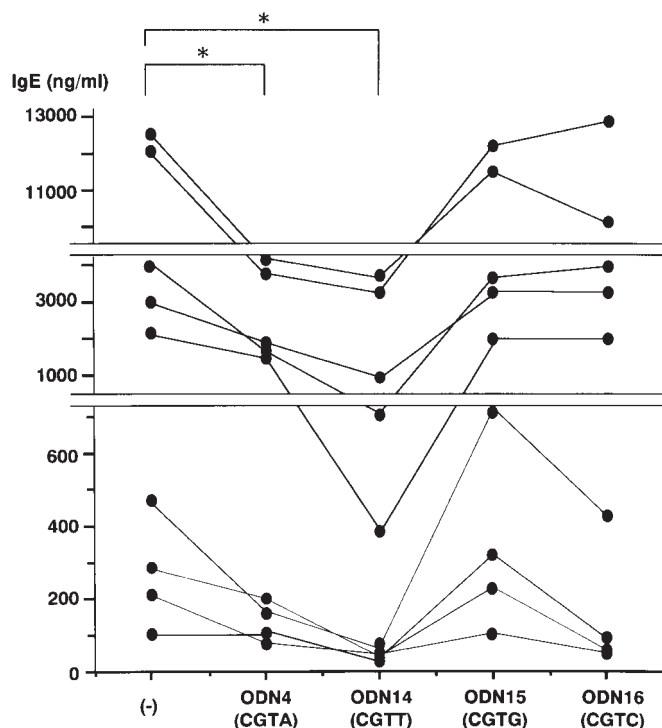


Figure 2. Comparison of IgE-suppression by four types of ODNs. Human PBMC from healthy donors (n = 9) were cultured for 14 d with IL-4 (100 U/ml) plus anti-CD40 mAb (0.1 μg/ml) in the presence of 5 μM ODN. The levels of IgE are expressed individually. ODN 14 inhibited IgE synthesis in all cases.

ODN 4 (CGTACG) with the 12 theoretically possible base combinations for these two sites. As shown in Table 3, ODN 14 and ODN 4 inhibited IgE synthesis by PBMC stimulated with IL-4 and anti-CD40 mAb. Addition of the other 14 ODNs to the IgE induction system had no effect on the measured IgE values. The inversion of C and G (ODN 41, GCTTCG) did not suppress IgE production. ODN 42, containing CGTTGC, also had no effect on IgE synthesis, suggesting that both CG sequences in ODN 4 (CGTACG) are crucial for IgE-suppressing activity.

Krieg and coworkers demonstrated that optimal CpG DNA for activation of mouse Th1 cytokine-producing cells is flanked by two 5' purines and two 3' pyrimidines (5, 6, 8). When we focused on the first CpG in the core sequence of ODN 14, CGTTCG, the flanking bases on the 5' side of the core sequence were AT (purine-pyrimidine), and those on the 3' side of the core sequence were TT (pyrimidine-pyrimidine). Thus, the flanking bases on the 3' side of the core sequence were consistent with the sequence proposed by Krieg and colleagues, but not those on the 5' side of the core sequence. We therefore replaced the 5' AT with the 15 theoretically possible nucleotide pairs for this site and investigated the effect of each resulting ODN on IgE synthesis. Five ODNs (ODN 14, ODN 27, ODN 31, ODN 35, and ODN 36) significantly inhibited IgE induction by PBMC stimulated with IL-4 and anti-CD40 mAb (Table 4). Only one combination (GACGTTTCG, ODN 31) was of the four possible purine-purine patterns in the 5' flanking location of the CpG, which was coincident with the sequence proposed by Krieg and coworkers, inhibited IgE synthesis. One 5' purine-pyrimidine pattern (ATCGTTCG, ODN 14), two pyrimidine-purine patterns (TACGTTTCG, ODN 27; CACGTT, ODN 36), and one pyrimidine-pyrimidine pattern (CTCGTTCG, ODN 35) resulted in potent ODNs. Thus, the eight bases NACGTTTCG or

TABLE 2

ROLE OF PALINDROMIC SEQUENCE IN THE SUPPRESSION OF IMMUNOGLOBULIN E PRODUCTION BY OLIGODEOXYNUCLEOTIDES

ODN		
No.	Sequence (5'→3')	IgE (ng/ml)
(-)	(-)	3,872 $\pm$ 1,654
4	accgat CGTACG gccgggtgacggcaccacg	2,089 $\pm$ 832*
14	T.....	1,437 $\pm$ 722†
15	G.....	3,885 $\pm$ 1,628
16	C.....	3,665 $\pm$ 1,564

Definition of abbreviations: mAb = monoclonal antibody; ODN = oligodeoxynucleotide; PBMC = peripheral blood mononuclear cell.

Human PBMC from healthy donors (n = 9) were cultured for 14 d with IL-4 (100 U/ml) plus anti-CD40 mAb (0.1 μg/ml) in the presence of 5 μM of ODN. The levels of IgE are expressed as mean $\pm$ SEM.

* p < 0.05 versus value in the absence of ODN.

† p < 0.01 versus value in the absence of ODN.

TABLE 3

ROLE OF 3' BASES OF CG SEQUENCES IN THE SUPPRESSION OF IMMUNOGLOBULIN E PRODUCTION BY OLIGODEOXYNUCLEOTIDES

ODN		
No.	Sequence (5'-3')	IgE (ng/ml)
(-)	(-)	2,134 ± 944
4	accgat CGTACG gccgggtgacggcaccacg	1,034 ± 380*
14	TT.....	776 ± 576†
15	TG.....	1,967 ± 1,033
16	TC.....	1,917 ± 993
5	GC.....	2,261 ± 1,128
11	GA.....	2,414 ± 1,523
12	GT.....	1,737 ± 895
13	GG.....	1,915 ± 966
10	AT.....	2,376 ± 1,105
18	AA.....	2,452 ± 1,615
19	AC.....	2,202 ± 1,597
20	AG.....	2,645 ± 1,785
17	CG.....	2,273 ± 1,198
21	CA.....	2,129 ± 1,338
22	CC.....	2,300 ± 1,482
23	CT.....	2,713 ± 1,843
41	GCTA.....	2,241 ± 1,120
42	TAGC.....	1,798 ± 988

Definition of abbreviations: mAb = monoclonal antibody; ODN = oligodeoxynucleotide; PBMC = peripheral blood mononuclear cell.

Human PBMC (n = 9) were cultured for 14 d with IL-4 (100 U/ml) plus anti-CD40 mAb (0.1 µg/ml) in the presence of 5 µM of ODN. IgE levels are expressed as mean ± SEM.

* p < 0.05 versus value in the absence of ODN.

† p < 0.01 versus value in the absence of ODN.

A/CTCGTTCG had the ability to inhibit IgE synthesis by human PBMC stimulated with IL-4 and anti-CD40 mAb.

ODNs Inhibited both IgE and IgG₄ Production by PBMC

To demonstrate the specificity of the ODN effect, we measured IgG₁, IgG₄, and IgA in PBMC culture supernatants (Table 5). The addition of ODN 4 or ODN 14 suppressed IgG₄ production by PBMC stimulated with IL-4 and anti-CD40 mAb. However, neither IgG₁ nor IgA production was influenced by either ODN 4 or ODN 14. ODN 15 and ODN 16 also had no influence on IgE, IgG₁, IgG₄, or IgA.

Inhibition of IgE Production by ODN is Mediated by IFN-γ and IL-12

Certain ODNs containing palindromic sequence have been shown to induce production of large amounts of IFN-γ by mouse spleen cells (12–14). This suggested the possibility that IFN-γ induced by ODN 14 blocked the production of IgE by human PBMC. We therefore measured the concentration of IFN-γ in the supernatants of 7-d cultures, using ELISA. The concentration of IFN-γ in supernatant from PBMC stimulated with IL-4 plus anti-CD40 in the presence of ODN 14 (5 µM) was significantly higher than that in the absence of ODN or in the presence of ODN 15 or ODN 16 (p < 0.01, respectively, Figure 3). Also, the addition of ODN 4 induced production of a large amount of IFN-γ by PBMC in the presence of IL-4 plus anti-CD40 (p < 0.05).

To confirm the involvement of IFN-γ in the inhibition of IgE production caused by ODN 14, we added an anti-IFN-γ mAb (1 µg/ml) to the culture system. The addition of the anti-IFN-γ mAb only partly blocked the inhibition of IgE production by ODN 14 in the presence of IL-4 plus anti-CD40 mAb (p < 0.05; Figure 4), whereas control mouse IgG had no effect on the IgE production (data not shown).

TABLE 4

ROLE OF 5' BASES OF CG SEQUENCES IN THE SUPPRESSION OF IMMUNOGLOBULIN E PRODUCTION BY OLIGODEOXYNUCLEOTIDES

ODN		
No.	Sequence (5'-3')	IgE (ng/ml)
(-)	(-)	5,142 ± 1,866
14	accgAT GGTTCG gccgggtgacggcaccacg	2,587 ± 1,040*
26	GG.....	4,915 ± 1,731
27	TA.....	2,758 ± 1,452*
28	CG.....	3,599 ± 1,507
29	AA.....	3,714 ± 1,382
30	AG.....	3,548 ± 1,615
31	GA.....	2,818 ± 1,510*
32	TT.....	3,281 ± 1,540
33	GC.....	5,037 ± 1,962
34	GT.....	3,883 ± 1,556
35	CT.....	2,939 ± 1,331*
36	CA.....	3,085 ± 1,560*
37	CC.....	3,654 ± 1,580
38	AC.....	5,078 ± 1,802
39	TC.....	4,642 ± 1,686
40	TG.....	4,308 ± 1,744

Definition of abbreviations: mAb = monoclonal antibody; ODN = oligodeoxynucleotide; PBMC = peripheral blood mononuclear cell.

Human PBMC (n = 8) were cultured for 14 d with IL-4 (100 U/ml) plus anti-CD40 mAb (0.1 µg/ml) in the presence of 5 µM of ODN. The IgE values are expressed as mean ± SEM.

* p < 0.05 versus value in the absence of ODN.

IL-12 enhances induction of IFN-γ by NK cells and T cells (21). The augmentation of IFN-γ induction by ODN 14 led us to investigate whether IL-12 is involved in the mechanism of IgE suppression by ODN 14. The addition of anti-IL-12 mAb at 10 µg/ml, a concentration that neutralizes the activity of approximately 5 ng of recombinant IL-12, also partly blocked the inhibition of IgE production by ODN 14, as had been the case with the anti-IFN-γ mAb (p < 0.05; Figure 4). It is known that IL-12 exerts pleiotropic effects via binding to the IL-12 receptor (IL-12R). However, no marked difference was detected in the level of IL-12Rβ expression by PBMC stimulated with IL-4 plus anti-CD40 mAb in the presence of ODN 14 and IL-12Rβ expression by PBMC stimulated in the absence of ODN 14, with either Western blotting or flow cytometry done with anti-IL-12Rβ (data not shown).

Inhibition of IgE Production in NK-Depleted Cell Fraction by ODN 14

CpG DNA promotes NK cell function both directly and indirectly, and enhances IFN-γ production by NK cells in mice (6). We obtained an isolated CD56⁺ cell fraction through use of

TABLE 5

ISOTYPE-SPECIFIC SUPPRESSION BY OLIGODEOXYNUCLEOTIDES

ODN No.	IgG ₁	IgG ₄	IgA
(-)	146.7 ± 18.6	30.2 ± 14.2	481.7 ± 102.9
4	167.8 ± 33.6	10.1 ± 2.8*	458.1 ± 100.7
14	146.2 ± 16.3	3.3 ± 1.5*	492.2 ± 111.1
15	171.5 ± 27.6	25.1 ± 13.1	528.5 ± 151.9
16	158.9 ± 25.1	30.6 ± 12.2	459.3 ± 108.0

Definition of abbreviations: mAb = monoclonal antibody; PBMC = peripheral blood mononuclear cell.

Human PBMC from healthy donors (n = 5) were cultured for 14 d with IL-4 (100 U/ml) plus anti-CD40 mAb (0.1 µg/ml) in the presence of 5 µM ODN. The levels of Ig are expressed as mean ± SEM (ng/ml).

* p < 0.05 versus value in the absence of ODN.

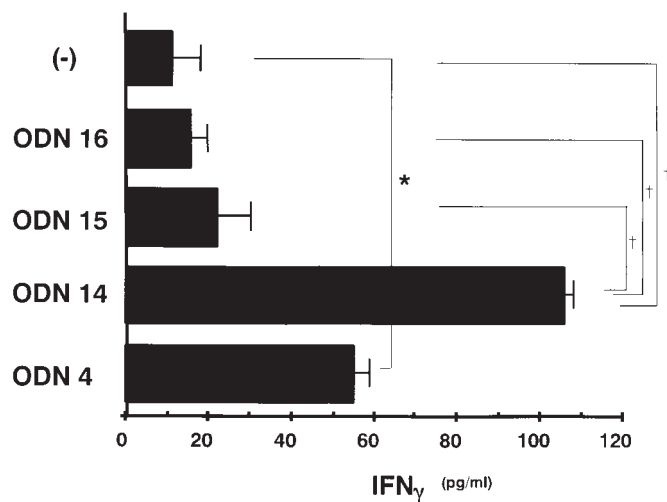


Figure 3. Production of IFN- γ . PBMC from healthy donors ($n = 6$) were stimulated with IL-4 (100 U/ml) and anti-CD40 mAb (0.1 μ g/ml) for 7 d in the absence or presence of ODN (5 μ M). Concentrations of IFN- γ in the supernatants were assessed with ELISA kits. Results are expressed as mean $\pm$ SEM (pg/ml). ODN 14 induced production of a significant amount of IFN- γ in human PBMC. * $p < 0.05$, $^{\dagger}p < 0.01$ compared with IFN- γ values for PBMC in the absence of ODN.

the MACS system. We also cultured a CD56 $^{-}$ cell fraction with IL-4 and anti-CD40 mAb in the presence of ODN 14 or ODN 15. As shown in Figure 5, the addition of ODN 14 inhibited IgE production by CD56 $^{-}$ cells but the addition of ODN 15 did not. However, the extent of ODN 14-induced IgE suppression in the CD56 $^{-}$ cell fraction was less than that in PBMC (Figure 1). Thus, CD56 $^{+}$ cells appear to be involved in the mechanism of IgE suppression by ODN, but other cells also contribute to the inhibition of IgE production by ODN.

The ODN Inhibited the Induction of ϵ Germline Transcripts

Expression of germline transcripts from Ig heavy-chain loci precedes isotype switching, and is thought to play an important role in Ig class switching (20). We assessed the presence

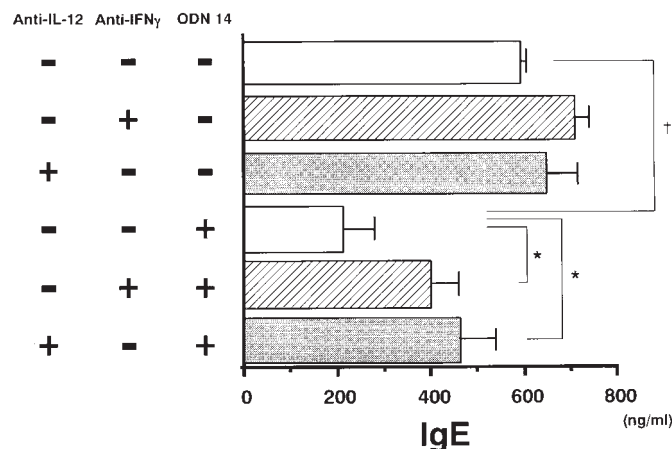


Figure 4. The addition of antibodies blocked the inhibition by ODN 14 of IgE production stimulated with IL-4 plus anti-CD mAb. PBMC from nonatopic healthy donors were cultured for 14 d with IL-4 (100 U/ml) and anti-CD40 mAb (0.1 μ g/ml) in the presence or absence of ODN 14 (5 μ M) and/or anti-IFN- γ mAb (1 μ g/ml), or anti-IL-12 mAb (10 μ g/ml). The addition of each antibody only partly blocked the inhibition of IgE production by ODN 14. Representative data from one of three experiments with similar results are shown. * $p < 0.05$, $^{\dagger}p < 0.01$.

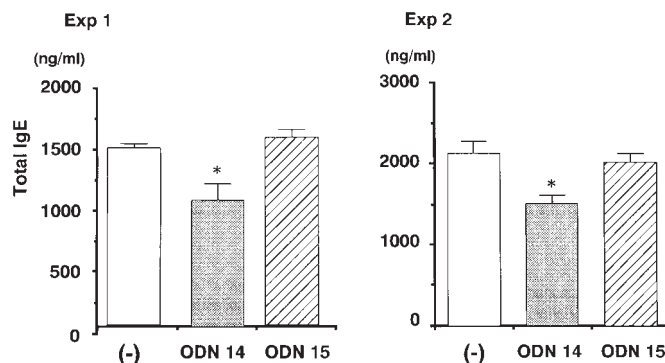


Figure 5. IgE synthesis in the CD56 $^{+}$ -depleted cell fraction was suppressed by the addition of ODN 14. The CD56 $^{+}$ -depleted cell fraction from nonatopic healthy PBMC was cultured for 14 d with IL-4 (100 U/ml) and anti-CD40 mAb (0.1 μ g/ml) in the presence or absence of ODN 14 (5 μ M) or ODN 15 (5 μ M). The CD56 $^{-}$ cells only partly blocked the suppression of IgE synthesis in the presence of the ODN 14.

of ϵ germline transcripts by using a reverse transcription(RT)-PCR strategy. As previously reported, stimulation with IL-4 reproducibly induced ϵ germline transcripts in PBMC (1, 20, Figure 6). The amplified fragments of ϵ germline transcripts were cloned and identified as ϵ germline transcripts through DNA sequencing analysis (20). Stimulation with ODN 14 and ODN 4 suppressed the expression of ϵ germline transcripts induced by IL-4 in all three experiments conducted with independent PBMC preparations. However, the addition of ODN 15 had no effect on the expression of ϵ germline transcripts. To estimate the sensitivity of our PCR assay for ϵ germline transcripts, we serially diluted plasmid DNA containing an I ϵ -C ϵ fragment (corresponding to ϵ germline transcripts) and subjected it to PCR amplification (20). Semiquantitative analysis, done by comparison of the data, demonstrated that the combination of ODN 14 and IL-4 induced only about one-tenth the amount of ϵ germline transcripts induced by IL-4 alone (data not shown). There was no difference in the staining intensity of the amplified bands of β -actin among five groups of PBMC whose ϵ germline transcript analyses are shown in Figure 6 (data not shown).

ODN Did Not Suppress IgE Production by IgE-Committed Cells

IgE production involves several stages, including isotype switching, differentiation, proliferation, and secretion. To examine the effect of ODN on IgE-committed cells, we cultured AF-10 cells that had already switched to IgE and were IgE-producing cells in the presence of four types of ODNs. The

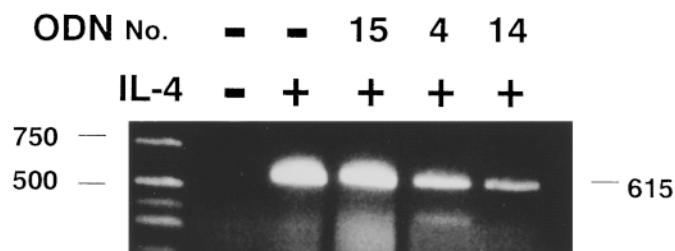


Figure 6. ODNs inhibited induction of ϵ germline transcripts by IL-4. Induction of ϵ germline transcripts from PBMC was assessed with RT-PCR. PBMC were cultured for 7 d with medium, IL-4 (100 U/ml) alone, or IL-4 (100 U/ml) plus ODNs (5 μ M). Cells stimulated with IL-4 expressed the predicted 615-bp band for ϵ germline transcripts. ODN 4 and ODN 14 inhibited the induction of ϵ germline transcripts.

four types of ODNs (ODN 4, ODN 14, ODN 15, and ODN 16) had no effect of IgE production by AF-10 cells (Figure 7A). We then examined delayed addition of ODN during IgE production after stimulation with IL-4 and anti-CD40 mAb, using atopic PBMC. Three patients, who had atopic dermatitis and nasal allergy, had high serum levels of total IgE ($> 5,000$ IU). Representative data from three independent atopic PBMC samples from these patients are shown in Figure 7B. The addition of ODN 14 inhibited IgE synthesis in all three atopic PBMC samples, as it did in nonatopic PBMC. In two of three experiments in which ODN 14 was added on Day +3 to B cells stimulated with IL-4 and anti-CD40 mAb, inhibition of IgE synthesis was observed. However, in all three experiments, the addition of ODN 14 on Day +7 did not suppress IgE synthesis.

DISCUSSION

In this study we showed that single-stranded synthetic ODNs containing NACGTTTCG or A/CTCGTTTCG sequences inhibited, in a dose-dependent manner, production of IgE *in vitro*

by human PBMC stimulated with IL-4 plus anti-CD40 mAb. The concentration of IFN- γ in the supernatants of cultures grown for 7 d in the presence of certain ODNs was higher than that in the supernatants of cells grown in the absence of ODNs. The addition of anti-IFN- γ mAb or anti-IL-12 mAb blocked the inhibition by ODN of IgE production, suggesting that both ODN-induced IFN- γ and ODN-induced IL-12 are crucial in the mechanism of ODN-induced suppression of IgE production. ODN had no effect on already IgE-switched cell lines, and the delayed addition of ODN on Day 7+ did not suppress IgE synthesis by atopic PBMC, suggesting that the phase of isotype switching to ϵ is very critical for the mechanism of IgE-suppression by ODN.

We identified immunopotential DNA sequences from the sequence encoding *M. bovis* BCG (12–14). *Mycobacterium tuberculosis* and its related-products (BCG) have long been investigated with regard to their ability to inhibit IgE responses or allergic reactions *in vitro* and *in vivo* (22). Intranasal BCG infection suppressed the ovalbumin-specific Th2 response in the lung (23). Infection by *M. tuberculosis* induced IL-12 production by human monocytes (24). Infection of mice with

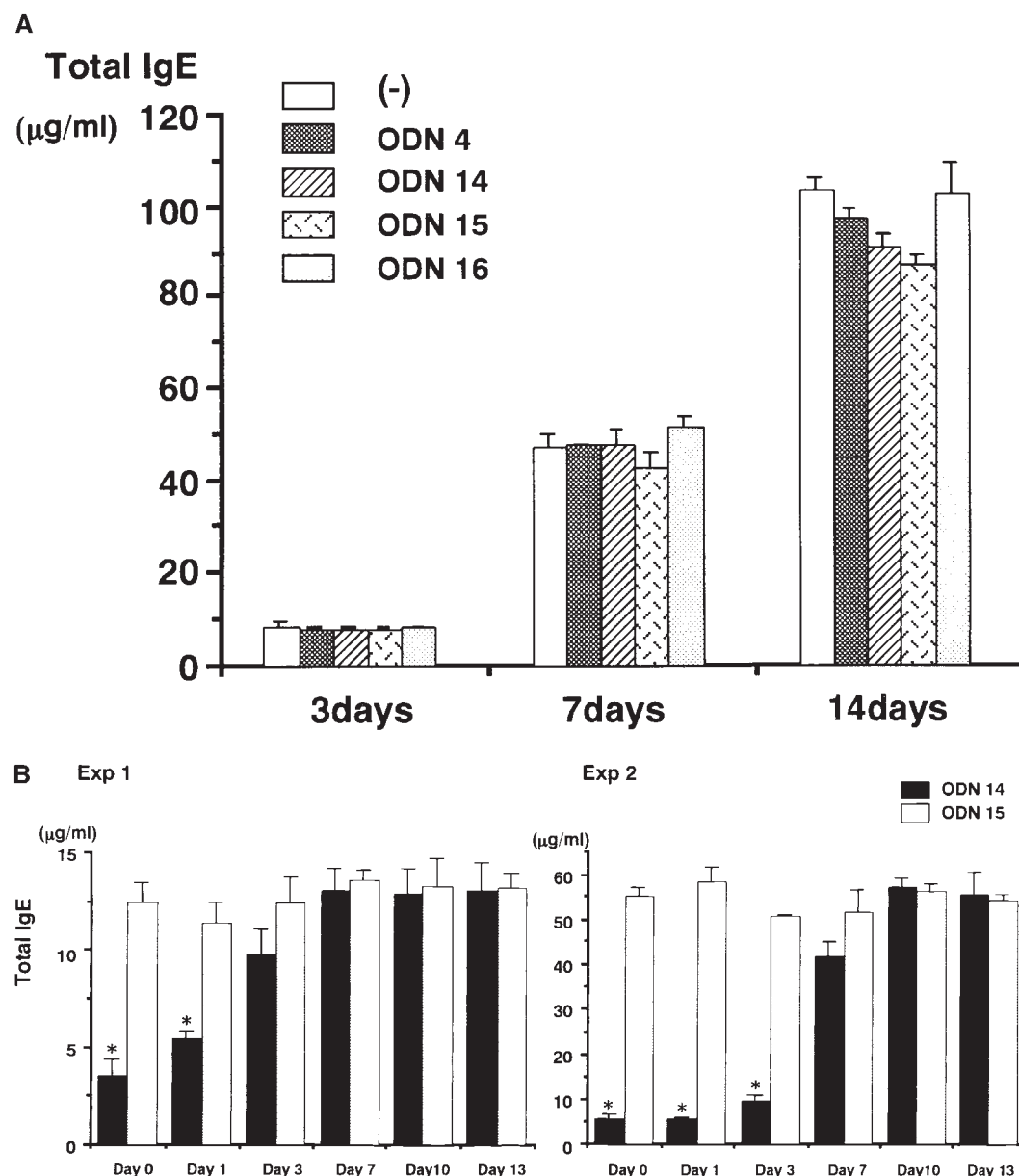


Figure 7. ODN had no effect on IgE synthesis by IgE-committed cells. (A) AF-10 cells, (from IgE-switched cell lines), were cultured in the presence of each ODN (5 μM) for 3, 7, or 14 d. There was no inhibition of IgE production in AF-10 cells by any ODN. (B) Delayed addition of ODN did not suppress IgE synthesis by atopic PBMC. PBMC from atopic donors were cultured with and without IL-4 (100 U/ml) and anti-CD40 mAb (0.1 $\mu\text{g/ml}$). ODN 14 or ODN 15 (5 μM), or an equal amount of plain medium, was added on Days 0, 1, 3, 7, 10, and 13. IgE production was measured on Day 14. Representative data from two of three experiments are shown. * $p < 0.01$.

BCG, through induction of IL-12, changed the cytokine secretion pattern of the CD4⁺ NK T-cell population from secretion of IL-4 to secretion of IFN- γ (25). An epidemiologic study demonstrated an inverse association between delayed hypersensitivity to *M. tuberculosis* and the incidence of atopic disease (26). Thus, the immunostimulatory sequence encoded in *M. bovis* BCG that we chose for the synthesis of ODNs may have far-reaching immunoregulatory significance.

With regard to the sequence of certain ODNs, the most effective sequence for IgE synthesis in human PBMC was that of ODN 14 (5'-accgATCGTTCGgccggtgacggcaccacg-3'). We determined that ODNs containing NACGTTTCG or A/CTCGTTCG sequences had strong activity in human PBMC. Krieg and associates stressed the need for a purine-purine (A or G)-CG-pyrimidine-pyrimidine (C or T) sequence for ODN activity in mice (5-11). Sonehara and coworkers demonstrated that the sequences showing the strongest ability to induce IFN in mice were NACGTN and NTCGAN, wherein N represents any complementary pair of bases (27). There are some discrepancies between their conclusion and our results, suggesting that the potent sequences for Th1 regulation may be different in humans and mice.

The molecular mechanisms by which certain ODNs mediate leukocyte activation are not clearly understood. The ODNs activate B cells (5, 7) and dendritic cells (28, 29), and augment T-cell responses to specific antigens (30). The stimulation of dendritic cells by ODNs is initiated by the uptake of the ODNs into endosomes. Endosomal maturation is required for subsequent activation of the stress kinase pathway, in which p38 kinase activation represents an essential step in ODN-triggered activation of antigen-presenting cells (APCs) (28, 29, 31). Activated APCs activate T-cells via costimulatory molecules and cytokine secretion. Activated T cells stimulate B cells via the CD40 system and cytokine secretion. Also, B cells are directly activated by the ODNs in a T-cell-independent manner (7). As this article was being completed, Bohle and associates reported that CpG DNA led to a significant increase in IFN- γ production both atopic and nonatopic human PBMC, and that IFN- γ production could be attributed to NK cells through an IL-12-dependent mechanism (32). Their results were partly coincident with our data. However, the depletion of CD56⁺ cells from PBMC did not completely block the suppression of IgE synthesis in the presence of the ODN used in our study. Since oligo-DNA containing CG palindromes act directly on human activated T cells to induce IFN- γ (33), T cells may be involved in the effect of the ODN on IgE synthesis. Further detailed studies are needed to evaluate possible interplay among the effects on immunopotent cells of stimulation with active ODNs.

Th1-type cytokines (IFN- γ and IL-12) are known to down-regulate IgE synthesis in human lymphocytes (1-3, 34). IL-12 may suppress IgE synthesis by two pathways: one an IFN- γ -dependent pathway and the other a novel IFN- γ -independent pathway (34). We found that the inhibition by ODNs of IgE production was not completely blocked by the addition of anti-IFN- γ mAb or anti-IL-12 mAb, suggesting that the ODNs may partly inhibit IgE production through a novel activity that is both IFN- γ - and IL-12-independent.

Systemic administration of immunostimulatory ODNs to mice led to transient splenomegaly and extramedullary hemopoiesis (35). Mice systemically injected with potent DNA showed a high serum concentration of TNF- α , suggesting that systemic administration of ODNs might cause septic shock (36). On the other hand, the intranasal administration of potent ODNs to mice inhibited IL-5 generation and induced allergen-specific IFN production without any adverse effects or

the risk of inducing active infection (17). We previously showed that repeated intranasal or intratracheal allergen challenge induced local *in vivo* ϵ isotype switching (37). Also, Chvatchko and coworkers demonstrated that repeated allergen challenges to sensitized mice caused the formation of germinal centers in the lung, which provided a local source of IgE-secreting cells (38). Additionally, we found that IgE values in nasal lavage fluid were associated with clinical outcome in the treatment of nasal allergy (39). These findings suggested that local administration of ODNs may be a reasonable therapeutic approach for respiratory allergic diseases. However, there has been one report of a severely detrimental side-effect of immunostimulatory ODNs. Schwartz and colleagues found that the intratracheal administration of potent ODNs to mice caused inflammation in the lower respiratory tract, raising the possibility that potent ODNs contribute to parenchymal damage to the lung (9). Also, the possibility that ODNs carry the risk of inducing cancer or unregulated cell growth cannot be completely ruled out. Although the local administration of ODNs in humans may be an effective strategy for treating allergic respiratory disease, the possible adverse effects of ODN should be thoroughly investigated before potent ODNs are used clinically.

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References

1. Gauchat, J. F., D. A. Lebman, R. L. Coffman, H. Gascan, and J. E. de Vries. 1990. Structure and expression of germline ϵ transcripts in human B cells induced by interleukin-4 to switch to IgE production. *J. Exp. Med.* 172:463-473.
2. Fujieda, S., P. A. Sieling, R. L. Modlin, and A. Saxon. 1998. CD1-restricted T-cells influence IgG subclass and IgE production. *J. Allergy Clin. Immunol.* 101:545-551.
3. Pène, J., F. Rousset, F. Brière, I. Chrétien, J.-Y. Bonnefoy, H. Spits, T. Yokota, N. Arai, K. Arai, J. Banchereau, and J. E. de Vries. 1988. IgE production by normal human lymphocytes is induced by interleukin-4 and suppressed by interferons- γ and - α and prostaglandin-E₂. *Proc. Natl. Acad. Sci. U.S.A.* 85:6880-6884.
4. Yamamoto S., T. Yamamoto, S. Shimada, E. Kuramoto, O. Yano, T. Kataoka, and T. Tokunaga. 1992. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. *Microbiol. Immunol.* 36:983-997.
5. Krieg, A. M., A.-K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546-549.
6. Klinman, D. M., A.-K. Yi, S. L. Beaucage, J. Conover, and A. M. Krieg. 1996. CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin-6, interleukin-12, and interferon- γ . *Proc. Natl. Acad. Sci. U.S.A.* 93:2879-2883.
7. Liang, H., Y. Nishioka, C. F. Reich, D. S. Pisetsky, and P. E. Lipsky. 1996. Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* 98:1119-1129.
8. Ballas, Z. K., W. L. Rasmussen, and A. M. Krieg. 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* 157:1840-1845.
9. Schwartz, D. A., T. J. Quinn, P. S. Thorne, S. Sayeed, A.-K. Yi, and A. M. Krieg. 1997. CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. *J. Clin. Invest.* 100:68-73.
10. Chu, R. S., O. S. Targoni, A. M. Krieg, P. V. Lehmann, and C. V. Harding. 1997. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J. Exp. Med.* 186:1623-1631.
11. Kline, J. N., T. J. Waldschmidt, T. R. Businga, J. E. Lemish, J. V. Weinstein, P. S. Thorne, and A. M. Krieg. 1998. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* 160:2555-2559.

12. Yamamoto, S., T. Yamamoto, T. Kataoka, E. Kuramoto, O. Yano, and T. Tokunaga. 1992. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J. Immunol.* 148:4072–4076.
13. Kuramoto, E., O. Yano, Y. Kimura, M. Baba, T. Makino, S. Yamamoto, T. Yamamoto, T. Kataoka, and T. Tokunaga. 1992. Oligonucleotide sequences required for natural killer cell activation. *Jpn. J. Cancer Res.* 83:1128–1131.
14. Tokunaga, T., O. Yano, E. Kuramoto, Y. Kimura, T. Yamamoto, T. Kataoka, and S. Yamamoto. 1992. Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of *Mycobacterium bovis* BCG induce interferons and activate natural killer cells. *Microbiol. Immunol.* 36:55–66.
15. Sato, Y., M. Roman, H. Tighe, D. Lee, M. Corr, M.-D. Nguyen, G. J. Silverman, M. Lotz, D. A. Carson, and E. Raz. 1996. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 273:352–354.
16. Roman, M., E. Martin-Orozco, J. S. Goodman, M.-D. Nguyen, Y. Sato, A. Ronaghy, R. S. Kornbluth, D. D. Richman, D. A. Carson, and E. Raz. 1997. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nature Med.* 3:849–854.
17. Broide, D., J. Schwarze, H. Tighe, T. Gifford, M.-D. Nguyen, S. Malek, J. V. Uden, E. Martin-Orozco, E. W. Gelfand, and E. Raz. 1998. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* 161:7054–7062.
18. Fujieda, S., S. Iho, Y. Kimura, H. Sunaga, H. Igawa, C. Sugimoto, S. Yamamoto, H. Saito. 1999. DNA from *Mycobacterium bovis* BCG (MY-1) inhibits IgE production by human lymphocytes. *Am. J. Respir. Crit. Care Med.* 160:2056–2061.
19. Fujieda, S., K. Zhang, and A. Saxon. 1995. IL-4 plus CD40 monoclonal antibody induces human B cell γ -subclass-specific isotype switching: switching to γ 1, γ 3, and γ 4, but not γ 2. *J. Immunol.* 155:2318.
20. Fujieda, S., Y. Q. Lin, A. Saxon, and K. Zhang. 1996. Multiple types of chimeric germ-line Ig heavy chain transcripts in human B cells: evidence for *trans*-splicing of human Ig RNA. *J. Immunol.* 157:3450–3459.
21. D'Andrea, A., M. Rengaraju, N. M. Valiante, J. Chehimi, M. Kubin, M. Aste, S. H. Chen, M. Kobayashi, D. Young, E. Nickbarg, R. Chizzonite, S. F. Wolf, and G. Trinchieri. 1992. Production of natural killer cell stimulatory factor (interleukin-12) by peripheral blood mononuclear cells. *J. Exp. Med.* 176:1387–1398.
22. Suemura, M., T. Kishimoto, Y. Hirai, and Y. Yamamura. 1977. Regulation of antibody response in different immunoglobulin classes: III. *In vitro* demonstration of "IgE class-specific" suppressor functions of DNP-*Mycobacterium*-primed T cells and the soluble factor released from these cells. *J. Immunol.* 119:149–155.
23. Erb, K. J., J. W. Holloway, A. Sobeck, H. Moll, and G. L. Gros. 1998. Infection of mice *Mycobacterium bovis*-Bacillus Calmette-Guérin (BCG) suppresses allergen-induced airway eosinophilia. *J. Exp. Med.* 187:561–569.
24. Fulton, S. A., J. M. Johnsen, S. F. Wolf, D. S. Sieburth, and W. H. Boom. 1996. Interleukin-12 production by human monocytes infected with *Mycobacterium tuberculosis*: role of phagocytosis. *Infect. Immun.* 64:2523–2531.
25. Emoto, M., Y. Emoto, I. B. Buchwalow, and S. H. E. Kaufmann. 1999. Induction of IFN- γ -producing CD4⁺ natural killer T cells by *Mycobacterium bovis* Bacillus Calmette-Guérin. *Eur. J. Immunol.* 29:650–659.
26. Shirakawa, T., T. Enomoto, S. Shimazu, and J. M. Hopkin. 1997. The inverse association between tuberculin responses and atopic disorder. *Science* 275:77–79.
27. Sonehara, K., H. Saito, E., Kuramoto, S. Yamamoto, T. Yamamoto, and T. Tokunaga. 1996. Hexamer palindromic oligonucleotides with 5'-CG-3' motif(s) induce production of interferon. *J. Interferon Cytokine Res.* 16:799–803.
28. Sparwasser, T., E.-S. Koch, R. M. Vabulas, K. Heeg, G. B. Lipford, J. W. Ellwart, and H. Wagner. 1998. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* 28:2045–2054.
29. Häcker, H., H. Mischak, T. Miethke, S. Liptay, R. Schmid, T. Sparwasser, K. Heeg, G. B. Lipford, and H. Wagner. 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* 17:6230–6240.
30. Sun S., H. Kishimoto, and J. Sprent. 1998. DNA as an adjuvant: capacity of insect DNA and synthetic oligodeoxynucleotides to augment T cell responses to specific antigen. *J. Exp. Med.* 187:1145–1150.
31. Yi, A.-K., and A. M. Krieg. 1998. Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J. Immunol.* 161:4493–4497.
32. Bohle, B., B. Jahn-Schmid, D. Maurer, D. Kraft, and C. Ebner. 1999. Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN- γ production in cells from allergic individuals and inhibit IgE synthesis *in vitro*. *Eur. J. Immunol.* 29:2344–2353.
33. Iho, S., T. Yamamoto, T. Takahashi, and S. Yamamoto. 1999. Oligodeoxynucleotides containing palindrome sequences with internal 5'-CpG-3' act directly on human NK and activated T cells to induce IFN- γ production *in vitro*. *J. Immunol.* 163:3642–3652.
34. Kishiwa, M., M. Gately, U. Gubler, R. Chizzonite, C. Fargeas, and G. Deslespesse. 1992. Recombinant interleukin-12 suppresses the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes. *J. Clin. Invest.* 90:262–266.
35. Sparwasser, T., L. Hültner, E. S. Koch, A. Luz, G. B. Lipford, and H. Wagner. 1999. Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. *J. Immunol.* 162:2368–2374.
36. Sparwasser, T., T. Miethke, G. Lipford, K. Borschert, H. Häcker, K. Heeg, and H. Wagner. 1997. Bacterial DNA causes septic shock. *Nature* 386:336–337.
37. Fujieda, S., D. Diaz-Sanchez, and A. Saxon. 1998. Combined nasal challenge with diesel exhaust particles and allergen induces *in vivo* IgE isotype switching. *Am. J. Respir. Cell Mol. Biol.* 19:507–512.
38. Chvatchko Y., M. H. Kosco-Vilbois, S. Herren, J. Lefort, and J.-Y. Bonnefoy. 1996. Germinal center formation and local immunoglobulin E (IgE) production in the lung after an airway antigen challenge. *J. Exp. Med.* 184:2353–2360.
39. Fujieda, S., I. Noda, H. Saito, K. Dejima, R. Kawata, N. Takagi, K. Saito, Y. Murakami, M. Imanaka, T. Terada, K. Lee, and H. Takenaka. 1998. Effect of pretreatment with Suplatast in patients with Japanese Sugi pollinosis: correlation with non-specific immunoglobulin E levels of serum and nasal lavage. In H. Stammberger and G. W. Monduzzi, editors. E.R.S. & I.S.I.A.N. Meeting '98, Italy. 155–159.