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Delineation of a CpG Phosphorothioate Oligodeoxynucleotide for Activating Primate Immune Responses In Vitro and In Vivo[1]

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Oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides within specific sequence contexts (CpG motifs) are detected, like bacterial or viral DNA, as a danger signal by the vertebrate immune system. CpG ODN synthesized with a nuclease-resistant phosphorothioate backbone have been shown to be potent Th1-directed adjuvants in mice, but these motifs have been relatively inactive on primate leukocytes in vitro. Moreover, in vitro assays that predict in vivo adjuvant activity for primates have not been reported. In the present study we tested a panel of CpG ODN for their in vitro and in vivo immune effects in mice and identified in vitro activation of B and NK cells as excellent predictors of in vivo adjuvant activity. Therefore, we tested >250 phosphorothioate ODN for their capacity to stimulate proliferation and CD86 expression of human B cells and to induce lytic activity and CD69 expression of human NK cells. These studies revealed that the sequence, number, and spacing of individual CpG motifs contribute to the immunostimulatory activity of a CpG phosphorothioate ODN. An ODN with a TpC dinucleotide at the 5′ end followed by three 6 mer CpG motifs (5′-GTCGTT-3′) separated by TpT dinucleotides consistently showed the highest activity for human, chimpanzee, and rhesus monkey leukocytes. Chimpanzees or monkeys vaccinated once against hepatitis B with this CpG ODN adjuvant developed 15 times higher anti-hepatitis B Ab titers than those receiving vaccine alone. In conclusion, we report an optimal human CpG motif for phosphorothioate ODN that is a candidate human vaccine adjuvant. *The Journal of Immunology,* 2000, 164: 1617–1624.

B acterial DNA, but not vertebrate DNA, has rapid immunostimulatory effects on leukocytes in vitro (1, 2). CpG dinucleotides are under-represented (CpG suppression, 1/50 to 1/60) and selectively methylated in vertebrate DNA, but are present at the expected frequency (1/16 bases) and unmethylated in bacterial DNA (3, 4). Teleologically, it appears likely that the recognition of unmethylated CpG dinucleotides within specific flanking bases (referred to as CpG motifs) may have evolved as an ancestral nonself pattern recognition mechanism used by the innate immune system to detect DNA of pathogens, such as bacteria and viruses (5). In mice the optimal CpG motif is an unmethylated CpG dinucleotide that is flanked by two 5′ purines and two 3′ pyrimidines, the best of which is 5′-GACGTT-3′ (6, 7). DNA containing these CpG motifs activates murine macrophages to secrete cytokines, especially IL-12, TNF-α, and IFN-$\alpha\beta$ (8–13), and stimulates murine dendritic cells (14, 15) and murine B cells (16–18). Acting in synergy with the CpG DNA (which does not directly stimulate highly purified NK cells), the IL-12 secondarily activates murine NK cells to secrete IFN-γ (10, 13) and to have increased lytic activity (9). Overall, CpG DNA induces a predominantly Th1 pattern of immune activation.

Synthetic oligodeoxynucleotides (ODN)[3] containing the optimal murine CpG motif (5′-GACGTT-3′) are known to be excellent immune adjuvants in various murine disease models and to drive Th1 immune responses (19–25). They are comparable or superior to CFA (but without apparent toxicity) and are superior to the standard human adjuvant alum with respect to the induction of Ag-specific humoral and cell-mediated immune responses (19–22, 25). Murine CpG ODN induce potent anti-tumor immune activity (26) and induce resistance to lethal challenge with *L. monocytogenes* (27). Our studies also support the use of CpG DNA for the conversion of allergic Th2-type immune responses into nonallergic Th1 responses (28).

Recently, we found that phosphorothioate ODN with the purine-purine-CG-pyrimidine-pyrimidine formula that had been identified as the most stimulatory motif in mice show no or only weak activity in human immune cells (29). We identified a potent human CpG motif, 5′-GTCGTT-3′, by testing phosphodiester ODN for the ability to stimulate human primary B cells (29) and found that

[3] Abbreviations used in this paper: ODN, oligodeoxynucleotides; HBsAg, hepatitis B surface Ag.

a phosphodiester ODN with a single copy of the optimal human CpG motif triggers ~60% of human peripheral blood B cells to proliferate and express high levels of CD86. We also demonstrated that this ODN, of sequence 2080, promotes growth, activation, and maturation of human peripheral blood dendritic cells (30).

To have in vivo clinical utility, ODN must be administered in a form that protects them against nuclease degradation. The native phosphodiester internucleotide linkage can be modified to become highly nuclease resistant via replacement of one of the nonbridging oxygen atoms with a sulfur, which constitutes phosphorothioate ODN. However, a phosphorothioate backbone reduces the affinity of the CpG ODN to a putative CpG binding protein(s) (B. Noll, W. Shen, C. Schetter, M. Wold, and A. M. Krieg, manuscript in preparation). Of note, an ODN containing a single optimal human CpG motif followed by a poly C tail, which is highly active with a phosphodiester backbone, is essentially inactive with a phosphorothioate backbone. In contrast, murine leukocytes are strongly activated by phosphorothioate ODN containing just one optimal murine motif. This argues for differences in the precise mechanism of CpG recognition between human and murine immune cells.

The goal of the present study was to identify the sequence of a human CpG phosphorothioate ODN that would have optimal adjuvant activity in vivo. Because we saw not only quantitative but also qualitative differences in the activities of different CpG ODN in mice, we first screened a panel of CpG and non-CpG control ODN on mouse cells to find in vitro assays with reliable and strong correlation to in vivo adjuvant activity with hepatitis B surface Ag (HBsAg). We then systematically tested a panel of >250 phosphorothioate ODN in corresponding human assays to identify sequences with in vitro immunostimulatory activity. We next examined whether the ODN with the highest activity in these human assays also activate B cell proliferation in chimpanzees and monkeys, and finally, whether they are active as adjuvants with HBsAg in chimpanzees and cynomolgus monkeys in vivo.

Materials and Methods

Oligodeoxynucleotides

Phosphorothioate-modified ODN were purchased from Operon Technologies (Alameda, CA) and Hybridon Specialty Products (Milford, MA). The sequences used are provided in Table I and Fig. 1. ODN were tested for endotoxin using the LAL-assay (BioWhittaker, Walkersville, MD; lower detection limit, 0.1 endotoxin units /ml). For in vitro assays, ODN were diluted in TE buffer (10 mM Tris, pH 7.0, and 1 mM EDTA) and stored at $-20°C$. For in vivo use, ODN were diluted in PBS (0.1 M PBS, pH 7.3) and stored at 4°C. All dilutions were conducted using pyrogen-free reagents.

Mouse spleen cell cultures

Spleens were removed from 6- to 12-wk-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME), 2×10^6 splenocytes were cultured with 0.2 μM ODN for 4 h (TNF-α) or 24 h (IL-6, IFN-γ, IL-12), and cytokines were detected by ELISA as previously described (16). To evaluate CpG-induced B cell proliferation, spleen cells were depleted of T cells with anti-Thy-1.2 and complement and centrifugation over Lympholyte M (Cedarlane Laboratories, Hornby, Canada), cultured for 44 h with the indicated ODN, and then pulsed for 4 h with 1 μCi of [³H]thymidine as described previously (5). To examine NK cell lytic activity, murine spleen cells were depleted of B cells using magnetic beads coated with goat anti-mouse Ig as previously detailed (31). Cells were cultured at 5×10^6/well in 24-well plates and harvested at 18 h for use as effector cells in a standard 4-h ⁵¹Cr release assay against YAC-1 target cells. One unit was defined as the number of cells needed to effect 30% specific lysis. ODN 1758 was also induced NK activity when tested at a higher concentration.

Immunization of mice against HBsAg and evaluation of the humoral response

Groups of 6- to 8-wk-old female BALB/c mice ($n = 5$ or 10; Charles River, Montreal, Canada) were immunized against HBsAg as previously de-

scribed (21). In brief, each mouse received a single i.m. injection of 50 μl of PBS containing 1 μg of recombinant HBsAg (Medix Biotech, Foster City, CA) and 10 μg of CpG ODN or non-CpG control ODN (see Table I for sequences) as a sole adjuvant or combined with alum (Alhydrogel "85," Superfos Biosector, Vedbaek, Denmark; 25 mg of Al³⁺/mg of HBsAg). Control mice were immunized with HBsAg without adjuvant or with alum. Plasma was recovered from mice at various times after immunization, and Abs specific to HBsAg (anti-HBs) were quantified by end-point dilution ELISA (in triplicate) as described previously (21). End-point titers were defined as the highest plasma dilution that resulted in an absorbance value (OD_{450}) 2 times higher than that of nonimmune plasma with a cut-off value of 0.05.

Isolation of primate PBMC and cell culture

PBMC were isolated from peripheral blood of healthy volunteers, chimpanzees, or rhesus or cynomolgus monkeys by Ficoll-Hypaque density gradient centrifugation (Histopaque-1077, Sigma, St. Louis, MO) as previously described (32). Cells were suspended in RPMI 1640 culture medium supplemented with 10% (v/v) heat-inactivated (56°C, 1 h) FCS (Hy-Clone, Logan, UT), 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies, Grand Island, NY; complete medium). Cells (final concentration, 1×10^6 cells/ml) were cultured in complete medium in a 5% CO_2 humidified incubator at 37°C. ODN and LPS (from Salmonella typhimurium; Sigma) or anti-IgM were used as stimuli. For measurement of human NK lytic activity, PBMC were incubated at 5×10^6/well in 24-well plates. Cultures were harvested after 24 h, and cells were used as effectors in a standard 4-h ⁵¹Cr release assay against K562 target cells as previously described (9, 31). For B cell proliferation, 1 μCi of [³H]thymidine was added 18 h before harvest, and the amount of [³H]thymidine incorporation was determined by scintillation counting on day 5. The SDs of the triplicate wells were <5%.

Flow cytometry on primate PBMC

Surface Ags on primate PBMC were stained as previously described (33). Monoclonal Abs to CD3 (UCHT1), CD14 (M5E2), CD19 (B43), CD56 (B159), CD69 (FN50), and CD86 (2331 (FUN-1)) were purchased from PharMingen (San Diego, CA). IgG1,κ (MOPC-21) and IgG2b,κ (30) were used to control for nonspecific staining. NK cells were identified by CD56 expression on CD3-, CD14-, and CD19-negative cells, whereas B cells were identified by expression of CD19. Flow cytometric data from 10,000 cells/sample were acquired on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). The viability of cells within the forward/side scatter gate used for analysis was examined by propidium iodide staining (2 μg/ml) and was > 98%. Data were analyzed using the computer program FlowJo (version 2.5.1, Tree Star, Stanford, CA).

Immunization of chimpanzees and cynomolgus monkeys against HBsAg and evaluation of the humoral response

Fourteen cynomolgus monkeys (2.0–3.5 kg) were immunized with a pediatric dose of Engerix-B (SmithKline Beecham Biologicals, Rixensart, Belgium) containing 10 μg of HBsAg adsorbed to alum (25 mg of Al³⁺/mg of HBsAg). This was administered alone ($n = 5$) or combined with CpG ODN 1968 ($n = 5$; 500 μg) or CpG ODN 2006 ($n = 4$; 150 μg; see Fig. 2 for sequences). Four chimpanzees (10–20 kg) were immunized in the same fashion, with two receiving control vaccine (Engerix-B only) and two receiving experimental vaccine (Engerix-B plus 1 mg of CpG ODN 2006). All vaccines were administered i.m. in the right anterior thigh in a total volume of 1 ml. Monkeys were maintained in the animal facility of the Primate Research Center (Bogor, Indonesia), and chimpanzees were housed at Bioqual (Rockville, MD). Animals were monitored daily by animal care specialists. No symptoms of general ill health or local adverse reactions at the injection site were noted. Plasma was recovered by i.v. puncture before and at various times after immunization and was stored frozen ($-20°C$) until assayed for Abs. Anti-HBs Abs were detected using a commercial ELISA kit (Monolisa Anti-HBs, Sanofi-Pasteur, Montreal, Canada), and titers were expressed in milliinternational units per milliliter based on comparison with World Health Organization-defined standards (Monolisa Anti-HBs Standards, Sanofi-Pasteur).

Results

Identification of CpG ODN with different profiles of in vitro immune activities

Our previous studies showed that the precise bases on the 5′ and 3′ sides of a CpG dinucleotide within a CpG motif have a major impact on the level of immune activation of a synthetic ODN, but

Table I. *Correlation of in vitro and in vivo CpG ODN immunostimulatory effects*

ODN	In Vitro[a]						In Vivo[b]	
	NK activity (LU)	B cell (SI)	IL-12 (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)	Anti-HBs, no alum	Anti-HBs + alum
Media	0	1	0	54	0	0	122	773
1982 (5′-TCCAGGACTTCTCTCAGGTT-3′)	0	1.9	0	0	0	2	54	774
1983 (5′-TTTTTTTTTTTTTTTTTTTT-3′)	0	1.2	183	223	0	28	66	638
1628 (5′-GGGGTCAA**CG**TTGAGGGGGG-3′)	0	6.0	417	377	0	303	501	2,480
1758 (5′-TCTCCCAGC**GT**GC**G**CCAT-3′)	0	14.0	2,995	214	82	28	564	1,650
1760 (5′-ATAAT**CG**A**CG**TTCAAGCAAG-3′)	2.0	25.7	3,612	631	302	144	1,372	3,574
1826 (5′-TCCATGA**CG**TTCCTGA**CG**TT-3′)	4.9	23.7	6,777	6,343	513	478	3,887	28,360
1841 (5′-TCCATAG**CG**TTCCTAG**CG**T-3′)	4.0	25.5	3,926	2,026	279	64	3,760	18,400
r vs anti-HBs (no alum)	0.98	0.84	0.88	0.85	0.90	0.57		
r vs anti-HBs (with alum)	0.95	0.70	0.86	0.91	0.88	0.68		

[a] In vitro assays were carried out on spleen cells removed from BALB/c mice as described in *Materials and Methods*. Stimulation index was determined as the ratio of cpm in wells without ODN to that in wells that had been stimulated throughout the culture period with the indicated ODN. Each in vitro value is the mean of triplicate assays.

[b] In vivo assays were carried out by immunization of BALB/c mice with 1 μg HBsAg plus 10 μg of the indicated ODN, with or without alum (25 μg Al^{3+}). Mice were bled at 4 wk postimmunization, and plasma was assayed for IgG anti-HBs Abs by ELISA assay. Anti-HBs values are the mean of titers obtained from 10 individual animals, and these are themselves the mean of duplicate assays.

it has been unclear whether different CpG motifs might display different immune effects. To evaluate this possibility, we tested a panel of CpG ODN for the ability to induce NK lytic activity and B cell proliferation and to stimulate synthesis of TNF-α, IL-6, IFN-γ, and IL-12 in murine spleen cells (Table I). Immunostimulatory activity of ODN without CpG motifs (ODN 1982 and ODN 1983; Table I) was negative or weak compared with that of CpG ODN. Consistent with our earlier findings (5) ODN with nonoptimal CpG motifs (ODN 1628 and ODN 1758) were less active than ODN containing CpG motifs flanked by two 5′ purines and two 3′ pyrimidines (ODN 1760, ODN 1826, and ODN 1841). Within these ODN, ODN 1826 containing two optimal murine CpG motifs (5′-GACGTT-3′) had the highest activity for five of six measured end points. Except for ODN 1628, all ODN showed a generally similar pattern of activity (NK cell-mediated lysis, B cell proliferation, IL-12, IL-6, TNF-α, and IFN-γ). Of note, ODN 1628, which was unique in this panel for containing two G-rich regions, showed preferential induction of IFN-γ synthesis but relatively low stimulation of the other activities.

Identification of in vitro assays that correlate with in vivo adjuvant activity

Because adjuvant activity is an in vivo end point, we were interested in identifying in vitro assays that would predict the adjuvant activity of a CpG ODN in vivo. The same ODN used for in vitro end points therefore were tested for their adjuvant activity to immunize mice against HBsAg. This was conducted both with ODN alone and with ODN combined with alum, since earlier studies had shown strong synergy for CpG ODN and alum adjuvants (21).

BALB/c mice immunized with HBsAg without adjuvant attained only low titers of anti-HBs by 4 wk, and this was not affected by addition of non-CpG control ODN. In contrast, addition of CpG ODN raised anti-HBs titers 5- to 40-fold depending on the sequence used (Table I). When alum was added, titers of anti-HBs were ~6 times higher than those with HBsAg alone. Nevertheless, the various ODN combined with alum gave similar levels of augmentation relative to alum alone, as was found with the nonalum formulations relative to no adjuvant. Specifically, non-CpG ODN had no effect, and the various CpG ODN augmented these titers 2- to 36-fold (Table I). Results obtained with the different ODN alone correlated very strongly (r = 0.96) with those obtained using the

same ODN plus alum. When linear regression was performed, a very high degree of correlation was found between certain in vitro assays and in vivo augmentation of anti-HBs titers. Of all the in vitro end points examined, the induction of NK lytic activity showed the best correlation to in vivo adjuvant activity (without alum, r = 0.98; with alum, r = 0.95; p < 0.0001). A good correlation regarding adjuvant activity was also obtained for B cell stimulation (r = 0.84 and 0.7) as well as secretion of TNF-α (r = 0.9 and 0.88), IL-12 (r = 0.88 and 0.86), and IL-6 (r = 0.85 and 0.91; Table I). The one in vitro assay that did not correlate well with the in vivo results was IFN-γ secretion (r = 0.57 and 0.68; Table I). This was due to the preferential IFN-γ-inducing activity of ODN 1628, which alone among the ODN in this panel contained G-rich regions. These data demonstrate that in vitro assays for NK lytic activity, B cell activation, and production of TNF-α, IL-6, and IL-12 provide valuable information in vitro to predict the adjuvant activity of a given ODN in vivo.

Screening of a phosphorothioate ODN panel to activate human NK cells

In previous studies we found that synthesis of inflammatory cytokines by human PBMC is induced by extremely low amounts of endotoxin (induced TNF-α secretion is detectable with just 6 pg/ml endotoxin, 2 logs more sensitive than murine immune cells) (34). In contrast, activation of human B cells and induction of human NK cell lytic activity with endotoxin are low even at high endotoxin concentrations. Based on these results we selected activation of NK cells (lytic activity and CD69 expression) and B cells (proliferation and CD86 expression) as the most highly specific and reproducible assays with low intersubject variability and used these assays for in vitro screening of a pool of ODN.

First we studied the effect of phosphorothioate ODN containing various combinations and permutations of CpG motifs on NK cell-mediated lysis of target cells. For clarity and ease of presentation, only data with selected representative CpG and control ODN are shown. Human PBMC were incubated with different phosphorothioate ODN (6 μg/ml) for 24 h and tested for their ability to lyse ^{51}Cr-labeled K562 cells. ODN without CpG motifs (ODN 1982 and ODN 2010; Fig. 1), runs of CpGs, ODN with nonoptimal CpG motifs, ODN containing only one 6-mer CpG motif (either 5′-GACGTT-3′ or 5′-GTCGTT-3′ underlined for clarity), and ODN

FIGURE 1. Induction of human NK cell lytic activity by phosphorothioate ODN. Human PBMC were incubated for 24 h with ODN (6 μg/ml) as indicated. Lysis of ^{51}Cr-labeled K562 cells was determined, and lytic units were calculated as described in *Materials and Methods*. Data represent the mean of two independent experiments with two different donors.
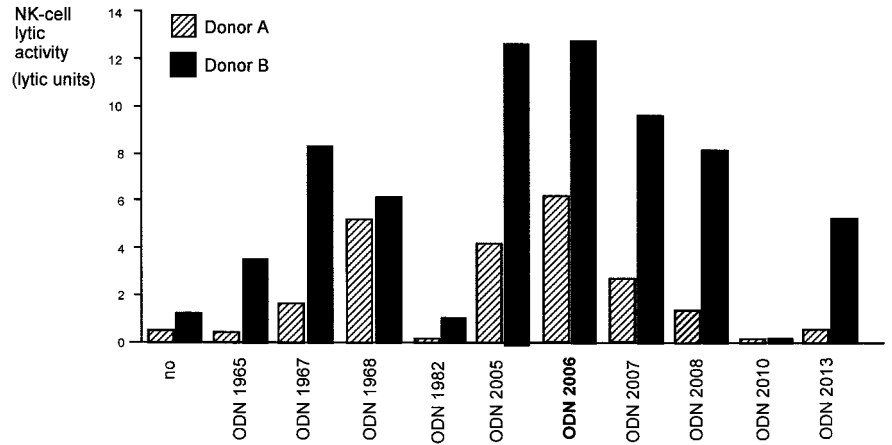
containing two of these motifs without a TpC on the 5′ end of the ODN failed to induce NK lytic activity substantially above background. Examples of such nonactive ODN with CpG motifs are ODN 1781 (5′-ACCATGGACGTTCTGTTTCCCCTC-3′), ODN 1823 (5′-GCATGACGTTGAGCT-3′), and ODN 1829 (5′-AT GACGTTCCTGACGTT-3′; not shown in figure). ODN with two 6-mer CpG motifs (either 5′-GACGTT-3′ or 5′-GTCGTT-3′) in combination with a TpC at the 5′ end of the ODN (ODN 1840, 5′-TCCATGTCGTTCCTGTCGTT-3′; ODN 1851, 5′-TCCT GACGTTCCTGACGTT-3′; not shown in figure) or with at least three 6-mer motifs without a TpC at the 5′ end (ODN 2013; Fig. 2) show intermediate activity. High activity was found when the 5′ TpC directly preceded a 6-mer human CpG motif (5′-TCGTCGTT-3′) and was followed by two 6-mer motifs (ODN 2005, ODN 2006, and ODN 2007). The best results were obtained when the 6-mer CpG motifs were separated from each other and from the 5′ 8-mer CpG motif by TpT (ODN 2006).

Expression of the activation marker CD69 is rapidly up-regulated on the surface of NK cells subsequent to stimulation. To confirm the results from the NK cell lysis assay, PBMC were incubated for 18 h with ODN (2 μg/ml). CD69 expression was examined on CD56-positive NK cells (CD3, CD14, and CD19 negative). Although induction of CD69 expression was less sequence restricted than stimulation of NK cell functional activity, control ODN (ODN 1982, ODN 2116, ODN 2117, and ODN 2010) showed only low activity similar to background levels (Fig. 2). ODN with two human CpG motifs separated by 5′-TTTT-3′ (ODN 1965) or four human CpG motifs without spacing (ODN 2013) were relatively more active at inducing CD69 expression (Fig. 2, *left panel*) than at stimulating NK cell lytic activity (Fig. 1). Optimal NK cell functional activity as well as CD69 expression were obtained with ODNs containing a TpC dinucleotide preceding the human CpG motif and additional human motifs within the sequence (ODN 2006 and ODN 2007; Fig. 2, *left panel*).
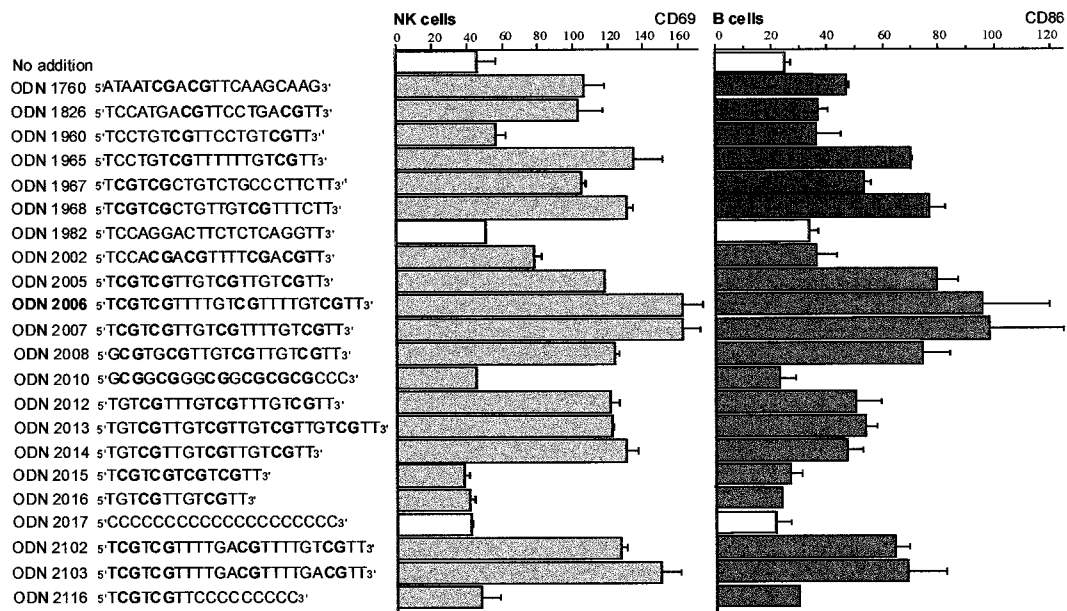
FIGURE 2. Screening for the optimal sequence of phosphorothioate ODN to activate human NK cells and B cells. Human PBMC were incubated with completely phosphorothioate-modified ODN (2 μg/ml for NK cells, *left panel*; 0.6 μg/ml for B cells, *right panel*) with the sequences indicated (CG dinucleotides in bold). CD69 expression was measured after 18 h on CD56-positive NK cells (negative for CD3, CD14, and CD19), and CD86 expression was measured after 48 h on CD19-positive B cells. The results show the means of experimental duplicates from two different donors for both NK cells and B cells.

Activity of phosphorothioate ODN for stimulating human B cells

In preliminary experiments we found that the percentage of proliferating B cells (5- (and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) assay, see *Materials and Methods*) correlated with the surface expression of the costimulatory CD86 on B cells, as measured by flow cytometry. Thus, we used CD86 expression on B cells to screen a panel of ODN for immunostimulatory activity. PBMC were incubated with 0.6 μg/ml ODN. Expression of CD86 (mean fluorescence intensity) was examined on CD19-positive B cells (Fig. 2, *right panel*). A poly C ODN (ODN 2017) or ODN without CpG dinucleotides (ODN 1982) failed to stimulate human B cells under these experimental conditions. A phosphorothioate ODN (ODN 2116) with one optimal human CpG motif preceded by a TpC (5′-TCGTCGTT-3′) was inactive (Fig. 2, *right panel*). The presence of one human 6-mer CpG motif (5′-GTCGTT-3′) had no activating effect (not shown). Two of these CpG motifs within the sequence showed no (ODN 1960 and ODN 2016) or intermediate (ODN 1965) activity dependent on the sequence context. If the ODN was composed of three or four copies of this motif (ODN 2012, ODN 2013, and ODN 2014), intermediate activity on B cells could be detected. The combination of the human 8-mer CpG motif on the 5′ end of the ODN with two 6-mer CpG motifs (ODN 2005, ODN 2006, ODN 2007, ODN 2102, and ODN 2103) led to a considerable increase in the ability of the ODN to stimulate B cells. The spacing between the single motifs was critical. The separation of CpG motifs by TpT was preferable (ODN 2006) compared with that of unseparated CpG motifs (ODN 2005; also compare ODN 1965 to ODN 1960). The human 6-mer CpG motif (5′-GTCGTT-3′) was better than the optimal mouse 6-mer CpG motif (5′-GACGTT-3′) when combined with the human 8-mer CpG motif on the 5′ end (ODN 2006 vs. ODN 2102 and ODN 2103). A (TCG)$_{poly}$ ODN was inactive or only weakly active, as were ODN containing CpG dinucleotides flanked by guanines or other CpG dinucleotides (ODN 2010; Fig. 2). Taken together, the findings for NK cells and B cells showed consistently that of the ODN tested, ODN 2006 has the highest immunostimulatory activity on human immune cells.

Comparative analysis of potency of CpG phosphorothioate ODNs in different primates

Different CpG motifs are optimal to activate murine and human immune cells. Furthermore, the number and location of CpG motifs within an active phosphorothioate ODN are different in mice and humans. We were interested to know whether CpG phosphorothioate ODN show similar activity among different species of primates. We compared a panel of CpG ODN for their ability to induce B cell proliferation in humans, chimpanzees, and rhesus or cynomolgus monkeys. The capability of ODN to stimulate human B cell proliferation (Table II) correlated well with their ability to

Table II. *Proliferative response of PBMC to phosphorothioate CpG ODN in primates*[a]

	Humans	Chimpanzee	Rhesus Monkey
No addition	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.0
ODN 1760	23 ± 7	0.3 ± 0.1	0.5 ± 0.3
ODN 1826	0.8 ± 0.1	0.4 ± 0.1	0.6 ± 0.1
ODN 1968	35 ± 9	20.0 ± 3.8	1.9 ± 0.7
ODN 1982	9.7 ± 1.1	2.5 ± 1.1	0.7 ± 0.1
ODN 2006	58 ± 8	27.4 ± 8.9	6.3 ± 3.3
ODN 2007	47 ± 11	0.5 ± 0.1	0.4 ± 0.2

[a] PBMC were prepared from peripheral blood and incubated with ODN (0.6 μg/ml) as indicated for 5 days. Proliferation was measured by uptake of [^{3}H]thymidine (cpm/1000) during the last 18 h. More than 95% of proliferating cells were B cells as determined using the 5- (and 6-) carboxy fluorescein diacetate succinimidyl (CFSE) assay. Four human probands, six chimpanzees, and two rhesus monkeys were tested. Standard error of means is indicated.

induce CD86 expression on B cells (Fig. 2, *right panel*). ODN 2006, which showed the highest activity in human B cells and NK cells, was also the most active in stimulating chimpanzee and rhesus monkey B cell proliferation (Table II). ODN 1968 and ODN 2006 gave the highest activation of cynomolgus monkey B cells in vitro (stimulation index, 25 and 29, respectively, at 6 μg ODN/ml). Surprisingly, CpG ODN 2007, which displayed similarly high activity as the optimal ODN 2006 in human cells, did not stimulate rhesus monkey or chimpanzee B cell proliferation, and ODN 1968 showed low activity. CpG ODN originally identified with high activity in mice (ODN 1760 and ODN 1826) showed little activity in monkeys (Table II).

In vivo adjuvant activity of CpG ODN in chimpanzees and cynomolgus monkeys

To evaluate whether CpG ODN with strong in vitro stimulatory effects on primate cells had detectable adjuvant activity in vivo, cynomolgus monkeys and chimpanzees were immunized with Engerix B, which comprises HBsAg adsorbed to alum, alone or with added ODN 1968 (500 μg) or ODN 2006 (1 mg), respectively. The results in the cynomolgus monkeys and chimpanzees cannot be directly compared because different CpG ODN were used. Nevertheless, compared with controls not receiving CpG ODN, anti-HBs titers at 4 wk postprime and 2 wk postboost were 66- and 16-fold higher, respectively, in the monkeys, and 15- and 3-fold higher in the chimpanzees (Table III). Thus, a clear adjuvant effect of CpG ODN was seen, and this was particularly striking after a single immunization. Because the number of animals studied is small, the differences seen are qualitative rather than quantitative.

Table III. *Anti-HBs responses in primates immunized against HBsAg with CpG ODN*[a]

Primate Species	n	CpG ODN	Anti-HBs (mIU/ml)	
			4 wk post-prime	2 wk post-boost
Cynomolgus monkey	5	None	15 ± 44	4,880 ± 13,113
	5	ODN 1968 (500 μg)	995 ± 1,309	76,449 ± 42,094
Chimpanzee	2	None	6, 11	3,712, 4,706
	2	ODN 2006 (1 mg)	125, 135	9,640, 16,800

[a] Animals were immunized by i.m. injection of Engerix B containing 10 μg HBsAg adsorbed to alum, alone or with added CpG ODN. Cynomologus monkeys were boosted at 10 wk and chimpanzees were boosted at 4 wk post-prime. Anti-HBs was determined by ELISA assay; values for monkeys are GMT ± SEM ($n = 5$), whereas individual values for the two chimpanzees in each group are provided.

Discussion

Previous studies have demonstrated that CpG ODN are superb vaccine adjuvants in mice (19–25). However, these mouse stimulatory ODN have shown more modest effects on human leukocytes (29). In this study we were interested in the identification of an optimal CpG ODN for use as a vaccine adjuvant in humans. In vivo screening of different CpG ODN for this purpose is not practical in primates, and for that matter, responses in nonhuman primates may not be predictive of in vivo effects in humans. Therefore, we wanted to identify an in vitro test that would predict in vivo efficacy. To do this, we evaluated several in vitro assays in mice for their predictive value of adjuvant activity in vivo in mice. CpG ODN-induced B cell and NK cell activation in murine spleen cells correlated particularly well with their ability to enhance the immune response against HBsAg. Based on the human CpG motif, 5′-GTCGTT-3′, previously identified using phosphodiester ODN, we designed a panel of CpG phosphorothioate ODN to identify the optimal sequence of a phosphorothioate ODN for activation of human B cells and NK cells. We found that the following characteristics contribute to the potency of a human CpG phosphorothioate ODN: 1) a TpC dinucleotide at the 5′ end followed by the human CpG motif (5′-GTCGTT-3′); 2) two additional human CpG motifs within the sequence; and 3) separation of adjacent CpG motifs by TpT. The ODN 2006, a 24 mer with three human CpG motifs, fulfills all three criteria and showed consistently the highest activity of all ODN tested. The ODN 2006 was an excellent adjuvant for the induction of an immune response in chimpanzees against HBsAg in vivo. In the presence of ODN 2006, anti-HBs titers at 4 wk after prime were more than 10-fold higher than levels considered to be protective (≥10 mIU/ml). In contrast, control chimpanzees receiving vaccine without ODN 2006 had anti-HBs near or below protective levels. In humans, the commercial vaccine, which contains alum as an adjuvant, is typically given in three doses. Although the majority of people respond well to two doses, the third dose ensures a very high (>90%) seroprotection rate.

The human stimulatory ODN 2006 shows weaker activity in mice compared with the highly active murine CpG ODN 1826 (our unpublished observations), supporting the concept of species specificity of CpG DNA recognition by immune cells. Although ODN 2006 was active in vitro in all primates tested, other CpG ODN, such as ODN 2007, had relatively high activity in human immune cells but no or a weaker effect in chimpanzees and rhesus monkeys. This argues for differences in the CpG recognition mechanism even within primates. Because bacterial DNA provides a large pool of CpG motifs, the evolutionary pressure to conserve the recognition mechanism for one single specific CpG motif is low. In contrast, the loss of binding of a transcription factor to its specific DNA motif would abolish its function. For the recognition of the presence of bacterial DNA, it, rather, makes evolutionary sense that different species develop their own optimized bacterial DNA recognition mechanism based on the bacterial environment to which they are exposed.

The good predictive value of B cell activation for in vivo vaccine adjuvant activity is most likely linked to the central role of B cells in the establishment of a specific immune response. Polyclonal proliferation of B cells (induced by CpG ODN) increases the likelihood of an Ag-specific B cell/Th cell match. Furthermore, enhanced expression of the costimulatory molecule CD86 on polyclonally expanded B cells activates Ag-specific Th cells. B cells also increase their CD40 expression in response to CpG ODN (29), improving the capability of CD40 ligand-expressing activated Th cells to stimulate B cells. Increased ICAM-1 synthesis on B cells facilitates the cell-to-cell contact (29). Thus, the activation status of polyclonal B cells plays a critical role during the initiation of a specific Ab response. The contribution of NK cell activity to the establishment of specific Abs is less obvious, and so the strong correlation between NK cell activation and in vivo adjuvant activity that was observed in Table I was unexpected. NK cells are part of the innate immune system and as such are involved in the first line of defense against pathogens. Most likely, the cytokine pattern produced by NK cells upon activation is closely related to the initiation of a specific immune response. Overall, IFN-γ secretion did not correlate well with in vivo adjuvant activity, but it remains possible that this may have contributed to the adjuvant activity of ODN 1628, especially because this ODN failed to induce any detectable NK lytic activity. The use of dendritic cell activation for the screening of CpG ODN may add valuable information in future studies and allow for the identification of CpG ODN other than or even more potent than ODN 2006.

As there is a 2-log higher endotoxin sensitivity of human than mouse primary monocytes, extreme caution is required to avoid endotoxin contamination of CpG DNA used for testing in the human system (34). Because TNF-α, IL-6, and IL-12 are produced by human monocytes in response to even low amounts of endotoxin, their value for high throughput in vitro screening assays is limited. On the other hand, human B cells and NK cells show only minor activation by endotoxin and thus are far more useful in testing for CpG DNA immunostimulatory activity.

Stimulation of cellular function in either NK or B cells (i.e., lytic activity, proliferation) requires a stronger CpG ODN than the induction of activation markers at their surface (CD69, CD86). For both cell types, the use of cell surface activation markers showed a higher nonspecific background, attributable to the phosphorothioate backbone, compared with the functional assays. This high sensitivity of surface markers requires the use of low ODN concentrations for optimal discrimination between ODN of similar activity. Thus, the use of surface markers allows the comparison of ODN with weak activity, while functional assays are preferred for comparing ODN with high activity. It is of note that the optimal ODN concentrations for stimulating B cells and NK cells differ. Although 0.6 μg/ml ODN is maximal to stimulate B cells, optimal NK cell activation may require 6 μg/ml ODN. Both B cell activation and NK cell functional activity are measured within freshly isolated PBMC. We found earlier that highly purified human primary B cells are activated by CpG DNA (29). The existence of a direct effect of CpG DNA on NK cells is less clear, and a secondary mechanism mediated by another cell type within PBMC might contribute to CpG-induced functional activity of NK cells.

It has been shown that some phosphorothioate ODN can induce human B cell proliferation (35, 36). Liang et al. found that the 6-mer 5′-TCGTCG-3′ at the 5′ end is critical for the activity of an ODN to stimulate human B cells at low concentrations, but that a (TCG)$_n$ ODN did not have higher activity. Poly T, A, C, or G ODN or ODN with a random sequence were not active in their assays. These results are in agreement with our findings. In addition, we demonstrate that the activity of an ODN is markedly enhanced if the 5′-TCGTCG-3′ is followed by TpT. This transforms the 5′-TCGTCG-3′ into a human CpG motif preceded by a TpC. This 8-mer motif followed by a poly C tail shows maximal activity if used as a phosphodiester ODN. but not as a phosphorothioate ODN (29). If a 5′ end 5′-TCGTCG-3′ is followed by another human CpG motif within the sequence, the activity is also increased. However, ODNs with three or four copies of the human CpG motif (5′-GTCGTT-3′) without the 5′-TCGTCGTT-3′ motif at the 5′ end showed only low activity. This is consistent with the study by Liang et al. (36), who tested ODNs with several copies of GACGTT, TGACGTT, or TGACGTC that were not particularly

potent. Consequently, combining the 5′-TCGTCGTT-3′ located at the 5′ end with additional 5′-GTCGTT-3′ motifs gave the best results in our study.

An important question is whether the immune effects of CpG motifs may be modified by the presence of other types of sequence motifs. To date, only two distinct immune effects of ODN sequences could be clearly identified: the effects due to CpG motifs and the effects due to G-rich motifs. We found a surprisingly poor correlation between ODN that induce strong IFN-γ secretion and those that are strong adjuvants. This poor correlation was largely due to the effects of ODN 1628, which was relatively weak as an adjuvant and at inducing secretion of most of the other cytokines, yet induced high level production of IFN-γ. A distinguishing feature of ODN 1628 is the presence of two G-rich regions, or poly G motifs, one of which has four Gs in a row, and the other of which has six Gs. Such poly G sequences show immunostimulatory effects that are distinct from CpG-mediated effects. For example, the level of IFN induction by a CpG ODN can be enhanced by poly G sequences at the ends of the same ODN (37). On the other hand, an ODN containing poly G sequences alone can block induction of IFN secretion by another ODN with a CpG motif (37). Poly G ODN can also block the production of IFN-γ induced by the mitogens Con A, bacterial DNA, or the combination of PMA and the calcium ionophore A23187 (44). This inhibition was only seen with the phosphorothioate backbone. Of note, poly G-rich ODN can also block the downstream effects of IFN-γ (38, 39). Also, we previously showed that the effects of poly G depend on the ODN backbone; poly G motifs increase the NK activity of chimeric ODN (phosphorothioate linkages on both ends, phosphodiester ODN in the middle), but reduce the NK activity of phosphorothioate ODN (9). Further studies will be required to determine whether the IFN-γ response to the poly G ODN 1628 is IL-12 independent and to identify the producing cell type.

Alum (e.g., Al_2O_3) was developed >75 yr ago and is still the only adjuvant approved for human use in most countries. Alum induces a Th2-type rather than a Th1-type immune response, appears to interfere with the development of cell-mediated immunity, and blocks activation of $CD8^+$ CTL (40). We showed earlier that CpG ODN induce a predominant IgG2a Ab response (Th1-like) to HBsAg in mice and, when the two adjuvants are used together, can even overcome the Th2 bias (IgG1) of alum for both Ab isotype and CTL responses (21). Furthermore, we found that the CpG ODN could induce HBsAg-specific CTL in young mice, in which a Th1 response normally is difficult to obtain (22, 41). Besides a shift toward a Th1 immune response, CpG ODN have the advantage over alum that it could be used as an adjuvant with live attenuated or multivalent vaccines that cannot be mixed with alum. In situations where it is necessary to overcome non- or hyporesponsiveness, the synergistic effect of CpG ODN and alum may be useful.

In preclinical studies antisense phosphorothioate ODN that are designed to inhibit target protein synthesis have been found to be safe at doses >100 mg/kg. In clinical antisense studies, phosphorothioate ODN have been used in doses up to 12 mg/kg with little drug-related toxicity (42, 43). This is ~30 times higher than the dose of 10 μg/mouse used in the present study (0.36 mg/kg). Phosphorothioate ODN can be produced on a large scale under good manufacturing practices conditions at a cost of approximately $200/g (42). Thus, the use of CpG ODN as adjuvant could significantly lower the cost of vaccination when repeated doses are normally required to induce a protective immunity (for example, with hepatitis B).

In conclusion, our study defines a CpG phosphorothioate ODN with high activity on human immune cells and with excellent adjuvant characteristics in chimpanzees. The design of this compound is based on the optimal human CpG motif and additional features that are essential if a phosphorothioate backbone is used. As a drug, ODN 2006 is inexpensive and easy to manufacture and is a candidate ODN for human clinical trials as an adjuvant for immunotherapy of cancer, infectious diseases, and allergy.

Acknowledgments

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