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Modulation of Oligonucleotide-Induced Immune Stimulation by Cyclodextrin Analogs

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ABSTRACT. Some phosphorothioate oligonucleotides have been shown previously to stimulate cell proliferation and immunoglobulin production. In the current study, we examined the effects of cyclodextrin analogs as immunomodulatory agents for oligonucleotide-induced immune stimulation, both *in vitro* and *in vivo*. Incubation of splenocytes with a 27-mer phosphorothioate oligonucleotide that induces immune stimulation increased cell proliferation as measured by [^{3}H]thymidine incorporation, whereas treatment of splenocytes with the phosphorothioate oligonucleotide complexed to cyclodextrin analogs markedly reduced oligonucleotide-induced cell proliferation. Similarly, administration of the 27-mer phosphorothioate oligonucleotide into mice resulted in splenomegaly and an increase in IgM production 48 hr post-administration. Administration of the oligonucleotide along with cyclodextrin analogs resulted in a significant suppression of splenomegaly and IgM response. Such suppression was dependent on the concentration of cyclodextrin analogs and was observed with various other immune stimulatory phosphorothioate oligonucleotide sequences. Administration of cyclodextrin analogs alone had no effect on splenomegaly or immune stimulation. BIOCHEM PHARMACOL 52;10:1537–1544, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. oligonucleotide; immune stimulation; cyclodextrin; immunomodulatory

Antisense oligonucleotides are short sequences of DNA that form specific hydrogen bonds with complementary single-stranded mRNA sequences or with double-stranded DNA, allowing regulation of specific foreign or cellular gene expression. They are widely used as research tools for inhibiting specific gene expression, and are under investigation for possible use as therapeutic agents [1, 2]. Human clinical trials using antisense oligonucleotides as therapy for diseases caused by HIV,† CMV, and HPV, and for cancer are already underway [3–5]. The success of the oligonucleotide therapy will depend upon the outcome of toxicological and pharmacological studies, as well as various other parameters. *In vivo* studies indicate that undesirable side-effects, such as immune stimulation, may occur following administration of some oligonucleotides. Injection of some phosphorothioate oligonucleotides into mice can cause massive splenomegaly and polyclonal hypergammaglobulinemia [6, 7]. The stimulatory effects depend on particular sequences of the oligonucleotide but do not depend on whether the oligonucleotides are antisense, sense, or scrambled with respect to their respective target genes. Certain sequence motifs or structures of oligonucleotides play important roles in inducing these immune responses. Kuramoto *et al.* [8] reported that the presence of particular palindromic sequences [e.g. 5′-CG-3′ motif(s)] induces natural killer cell activation. Krieg *et al.* [9] found that B cell activation requires cellular uptake of a DNA motif in which an unmethylated CpG dinucleotide is flanked by two 5′ purines and two 3′ pyrimidines. Methylation of the CpG with 5-methylcytosine reduces the immune stimulation of oligonucleotides. More recently, we have shown that chemical modifications of the oligonucleotides can reduce their immunostimulatory effect [10].

Cyclodextrin comprises a group of cyclic polysaccharides containing 6–8 glucose units with a cone-like cavity inside. The central cavity of the cyclodextrin molecule is hydrophobic, which provides opportunities for unique association with other molecules. The peripheral structure contains several hydroxyl groups, making the molecule water soluble. Parent cyclodextrins and various substituted analogs have been studied extensively as carriers for pharmaceuticals because of their unique characteristics [reviewed in Refs. 11 and 12]. Some cyclodextrin analogs, e.g. hydroxypropyl, hydroxyethyl, and methylated β-cyclodextrin, can enhance the solubility and bioavailability of a variety of pharmacological agents. Some analogs can also alleviate undesirable characteristics of drugs to which they are being complexed, such as by reducing local inflammation and protecting against drug-induced nephrotoxicity [13, 14]. Cyclodextrin analogs can be administered easily through oral, nasal, corneal, or rectal absorption and by intravenous, intramuscular, or transdermal injection [13–16].

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† *Abbreviations:* HBSS, Hanks' Balanced Salt Solution; HECD, hydroxylethyl cyclodextrin; HPCD, 2-hydroxypropyl cyclodextrin; HIV, human immunodeficiency virus; CMV, human cytomegalovirus; HPV, human papilloma virus; Ig, immunoglobulin; and PS, phosphorothioate.

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In the work presented here, we have explored the possibility of using cyclodextrin analogs to modulate the effects of phosphorothioate oligodeoxynucleotides on immune stimulation. The studies were carried out both *in vitro* and *in vivo*, using phosphorothioate oligodeoxynucleotides that have been reported previously to induce immune stimulation [6, 9].

MATERIALS AND METHODS

Synthesis of Oligonucleotides

PS-oligonucleotides were synthesized on an automated synthesizer (Millipore, 8700, Bedford, MA) using phosphoramidite chemistry [17]. The sequences of the PS-oligonucleotides used in the present study are oligo 1: 5′-TCG TCG CTG TCT CCG CTT CTT CTT GCC-3′ [27-mer] and oligo 2: 5′-GAG AAC GCT CGA CCT TCG AT-3′ [20-mer]. The oligonucleotides were deprotected by treatment with concentrated ammonium hydroxide at 55° for 12 hr and were purified by reverse-phase HPLC as described earlier [18].

In vitro *Experiments*

Spleens were removed from male CD1 mice (4–6 weeks; Charles River, Wilmington, MA). Single cell suspensions were prepared by gently mincing the spleens with frosted ends of glass slides. The cells were washed twice and then resuspended in RPMI complete medium [RPMI 1640 medium supplemented with 10% heat-inactivated (56° for 30 min) fetal bovine serum, 2 mM glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, and 50 μM 2-mercaptoethanol]. The cells were then plated in 96-well dishes at a density of 10^6 cells/mL, in a final volume of 100 μL. Oligonucleotides were added to the cell culture in 10 μL of TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA). Cyclodextrin analogs [HPCD (degree of substitution 5-8), HECD (degree of substitution 15), and Encapsin (HPCD with a degree of substitution of 17.8) (purchased pyrogen-free from Amaizo, Hammond, IN)] were added to the cells at a concentration of 0.5% in RPMI medium except where indicated. Cells were then cultured in a 37°, 5% CO_2–95% O_2, humidified air incubator for further studies. The experiments were performed in triplicate.

In vivo *Experiments*

PREPARATION OF OLIGONUCLEOTIDE–CYCLODEXTRIN COMPLEXES. Oligonucleotides (final concentration 4 mg/mL) were mixed with a 5% solution of one of the cyclodextrin analogs (HPCD or HECD) in sterile PBS, sonicated at 4° for 1 hr, and incubated at 4° overnight.

Male CD1 mice (4–5 weeks, 20–22 g; Charles River) were used in this study. The animals were fed with commercial diet and water *ad lib.* and were kept at the animal facility of the University of Massachusetts Medical Center (Worcester, MA). Oligonucleotide (1 mg oligo 1 or 0.5 mg oligo 2) in plain PBS or complexed with either 5% HPCD or 5% HECD in PBS were injected into mice intraperitoneally, in a volume of 250 μL. After 24 hr (in the case of oligo 2) or 48 hr (in the case of oligo 1), mice were killed, and spleens were harvested and prepared for further studies. Three to four mice were studied for each condition and were analyzed individually.

Thymidine Incorporation

Murine splenic lymphocytes (10^6 cells/mL) were cultured in the presence of oligonucleotides and/or cyclodextrin analogs, except where indicated. After 48 hr (or 24 and 72 hr in one experiment), cells were pulsed-labeled with 1 μCi [^{3}H]thymidine/well (in 10 μL of RPMI medium) for 4 hr. Cells were then harvested using an automatic cell harvester (Skatron, Sterling, VA), and the filters were counted using a scintillation counter. The experiments were performed in triplicate.

Cell Cycle Analysis

Spleens from *in vivo* treated mice were harvested, and single cell suspensions were cultured in RPMI complete medium (10^6 cells/tube in 1 mL RPMI). After 4 hr of incubation, cells were washed with FACS buffer (1× HBSS supplemented with 1% BSA and 0.1% sodium azide), fixed with 70% cold alcohol with immediate mixing, and then kept on ice for 30 min. After fixation, cells were washed twice and resuspended with 200 μL PBS and treated with 50 μL RNAse (10 mg/mL, DNase-free) at 37° for 30 min. Propidium iodide (50 μg/mL) was added to the cells before flow cytometry analysis. Flow cytometric data on 10,000 viable cells were acquired on an Epics XL flow cytometer (Coulter, Hialeah, FL). Data were analyzed by forward scatter versus side scatter, excluding doublets, using Epics XL, version 1.5, software and multicycle software (Phoenix Flow Systems, San Diego, CA). The experiments were performed in quadruplicate.

Immunoglobulin Synthesis

Spleens from *in vivo* treated mice were harvested, and single cell suspensions were incubated in culture at a density of 10^6 cells/mL, in 1 mL of RPMI complete medium. After 24 hr of incubation, cell culture supernatants were harvested by centrifugation, and the supernatants were assayed for IgM level using a standard ELISA. ELISA plates (96 wells) were coated with goat anti-mouse IgM (5 μg/mL) diluted in PBS supplemented with 0.05% sodium azide (pH 9.6) overnight at 4° and washed three times with PBS-T buffer (PBS supplemented with 0.05% Tween 20 and 0.25% BSA). Cell culture supernatants were added to the ELISA plates and incubated at 37° for 2 hr. Mouse IgM (1 mg/mL) was diluted with PBS-T to provide a standard curve between 0 and 800 ng/mL. The plates were washed three times with PBS-T buffer and incubated at 37° for 2 hr with goat anti-

mouse IgM conjugated to alkaline phosphatase diluted 1: 1000 with PBS-T buffer. The plates were again washed three times with PBS-T buffer, phosphate substrate (*p*-nitrophenylphosphate) in diethanolamine buffer (75 μL) was added, and the plates were kept at room temperature for 1 hr. The colorimetric reaction was stopped by the addition of 25 μL of 0.5 M sodium hydroxide. The optical density (405 nm) was measured using an automatic ELISA plate reader (Bio-Tek Instruments, Inc). IgM levels were calculated based on the standard curve. The experiments were performed in quadruplicate.

Statistical Analysis

Data are presented as means ± SD. Statistical significance between groups was determined using Student's *t*-test.

RESULTS

In vitro *Studies*

Recently, we reported that cyclodextrin analogs can increase cellular uptake of oligonucleotides [19, 20]. To further explore the characteristics of cyclodextrin analogs, we assessed their effects on immune stimulation induced by oligonucleotides both *in vitro* and *in vivo*. Some phosphorothioate oligonucleotides have been shown to induce cell proliferation and immune stimulation when incubated with splenocytes or when injected in mice. In this report, we investigated whether cyclodextrin analogs can modulate the immunostimulatory effect of oligonucleotides. Spleen lymphocytes were incubated *in vitro* with either oligonucleotides alone or in the presence of cyclodextrin analogs, and cell proliferation was measured by [^{3}H]thymidine incorporation. To induce proliferation of murine splenic lymphocytes, we used oligo 1, a 27-mer phosphorothioate complementary to the *rev* region of HIV-1, which has been shown to have immunostimulatory effects both *in vitro* and *in vivo* [6]. Figure 1 shows that after a 48-hr incubation, the effect of oligo 1 on cell proliferation was concentration dependent. The thymidine uptake increased by 5-fold at 1 μg/mL, and by 10-fold at 10 μg/mL compared with that of medium alone. When the oligonucleotide was incubated with splenocytes in the presence of 0.5% of either HPCD, HECD, or Encapsin, the level of cell proliferation decreased. At 1 μg/mL, the levels of cell proliferation were reduced to almost the basal level, and at 10 μg/mL the levels were reduced by approximately 30%. The suppression of cell stimulation was concentration dependent since higher concentrations of cyclodextrin analogs resulted in better suppression (data not shown). All three cyclodextrin analogs reduced cell proliferation to similar levels. There was no significant change in [^{3}H]thymidine incorporation when cyclodextrin analogs were used alone, indicating that cyclodextrin analogs by themselves do not suppress or induce stimulation of cell proliferation under this condition.

Time–course studies showed that the immunomodulatory effect of cyclodextrin analogs was dependent on the

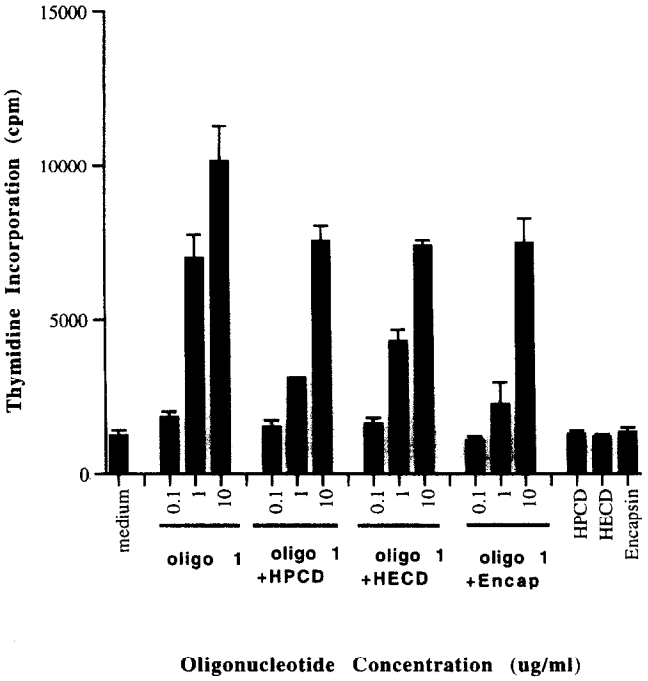

FIG. 1. Effect of cyclodextrin analogs on cell proliferation induced by oligo 1. Spleen cells were incubated with either free oligo 1 or complexed with 0.5% of cyclodextrin analogs (HPCD, HECD, and Encapsin). After 48 hr of incubation, cells were pulse-labeled with [^{3}H]thymidine. Results are means ± SD of triplicate experiments.

level of cell stimulation induced by oligonucleotide (Fig. 2). At 24 hr of incubation, there was no dramatic change in cell proliferation induced by oligo 1 either alone or in the presence of cyclodextrin analogs. At 72 hr of incubation, however, cell proliferation was increased greatly in the presence of the oligonucleotide and was reduced markedly in the presence of cyclodextrin analogs. We also examined the effects of cyclodextrin analogs and oligonucleotides on cell proliferation after 48 hr by cell cycle analysis using flow cytometry. Oligo 1 induced a significant increase in the percentage of cells in the S/G_2 phases (44.8% increase compared with the control). When 0.5% cyclodextrin analogs were added together with oligo 1, the percentage of cells in the S/G_2 phases was reduced significantly (11–16.8% increase compared with the control). These results suggest that cyclodextrin analogs can reduce the stimulation of cell proliferation induced by oligo 1.

We used the cyclodextrin analogs at a 0.5% concentration because in our previous studies we showed that these analogs, when used at 0.5%, can increase cellular uptake of oligonucleotides without inducing any cytotoxicity in cell culture [19]. We tested the effect of different concentrations of cyclodextrin analogs (0.1 to 0.5%) on stimulation of cell proliferation and found that the immunosuppressive effects were concentration dependent. At cyclodextrin analog concentrations of 0.5%, better suppression effects on cell proliferation were obtained than at lower concentrations (such as 0.2 or 0.1%) (data not shown). Incubation of

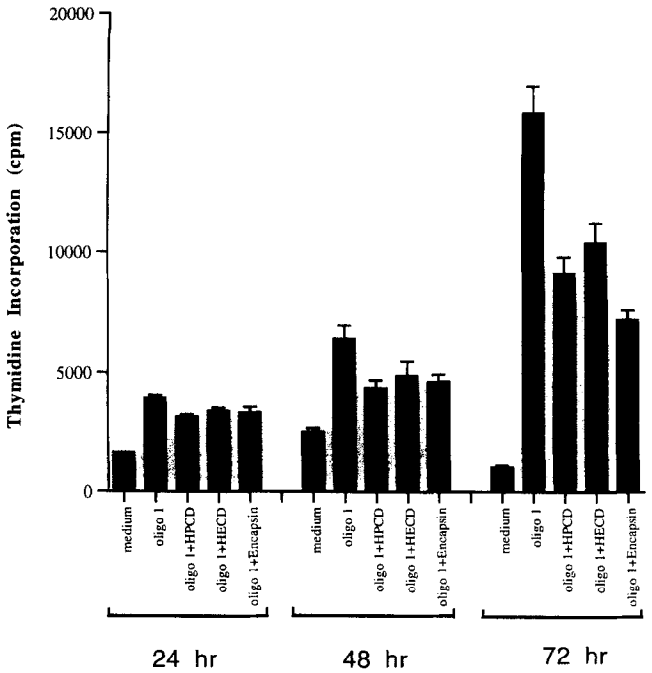

FIG. 2. Time–course effect of cyclodextrin analogs. Spleen cells were incubated with oligo 1 (10 μg/mL) either alone or in the presence of cyclodextrin analogs (HPCD, HECD, and Encapsin) (0.5%). After 24, 48, and 72 hr of incubation, cells were pulse-labeled with [^{3}H]thymidine. Results are means ± SD of triplicate experiments.

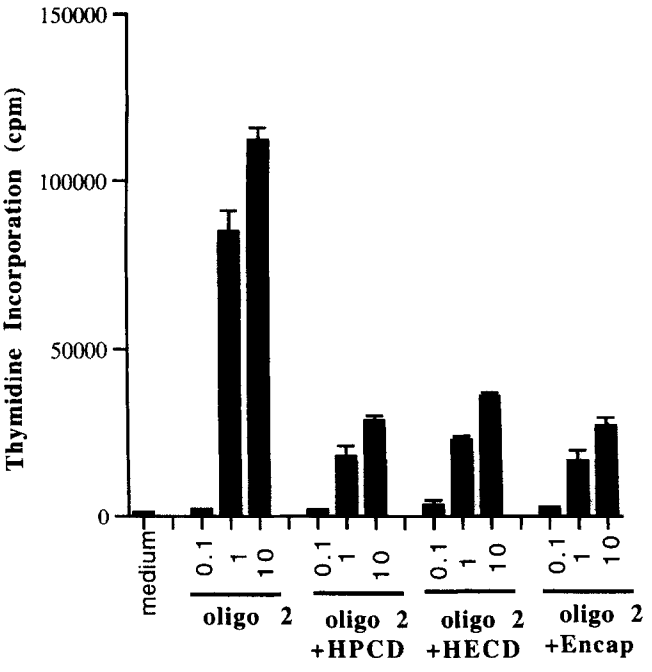

FIG. 3. Effect of cyclodextrin analogs on cell proliferation induced by oligo 2. Spleen cells were incubated with oligo 2 (10 μg/mL) either alone or in the presence of cyclodextrin analogs (HPCD, HECD, and Encapsin) (0.5%). Cell proliferation was measured 48 hr later by [^{3}H]thymidine incorporation. Results are means ± SD of triplicate experiments.

the cells with cyclodextrin analogs at concentrations higher than 1% results in cytotoxicity [19].

To rule out that the immunosuppressive effects of cyclodextrin analogs are not confined to oligo 1, we tested another oligonucleotide sequence with cyclodextrin analogs. Oligo 2 is a 20-mer phosphorothioate oligonucleotide that contains multiple 5′-CG-3′ motifs, and has been shown to have strong immune reaction both *in vitro* and *in vivo* [9]. Figure 3 shows that cells treated with 1 or 10 μg/mL of oligo 2 alone underwent a dramatic increase in cell proliferation. When cyclodextrin analogs (0.5%) were included in the cell culture, the increase in cell proliferation induced by oligo 2 was reduced significantly. We also assessed the effects of cyclodextrin analogs on various other immunostimulatory phosphorothioate oligonucleotide sequences, and similar results were observed (data not shown). These results suggest that the suppressor effect of cyclodextrin analogs is not restricted to one oligonucleotide sequence. Cyclodextrin analogs may be used, therefore, in conjunction with a wide range of oligonucleotides to modulate their immunostimulatory effects.

In vivo *Studies*

We tested whether the immunomodulatory effects of cyclodextrin analogs that were observed *in vitro* also occurred *in vivo*. CD1 mice were injected intraperitoneally with 50 mg/kg of oligo 1 in a volume of 250 μL PBS, either alone or complexed with HPCD at 2.5, 5, or 10% of the injection volume (which corresponds to 312, 625, or 1250 mg/kg, respectively). After 48 hr, the animals were euthanized, and spleens were harvested. The spleens from oligo 1-treated mice were about 1.5 times heavier than those from PBS-treated mice (Fig. 4). There was no significant reduction in spleen weight when 2.5% HPCD was used together with oligo 1. However, when HPCD was used at 5 or 10% together with oligo 1, there was significant decrease in spleen weight compared with oligo 1 alone. Administration of HPCD at the same concentrations (2.5, 5, and 10%) had no effect on spleen weight compared with PBS-treated mice.

To further investigate the difference in splenomegaly observed following injection of oligo 1 and the cyclodextrin analogs, we made single cell suspension of the spleens and cultured the cells for 4 hr for cell cycle analysis using flow cytometry. As shown in Table 1, there was an approximately 30% increase in the percentage of cells in G_2/S phases in oligo 1-treated mice compared with PBS-treated mice. Mice who received injection of HPCD along with the oligo 1 had less cycling cells than oligo 1-treated mice. This decrease was dependent on the concentration of HPCD: at 5 and 10% HPCD, the levels of the cells in G_2/S were similar to those in mice who received vehicle alone. Injection of HPCD alone (2.5, 5, and 10%) had no significant effect on cell cycle.

Similarly, when the spleen lymphocytes were measured

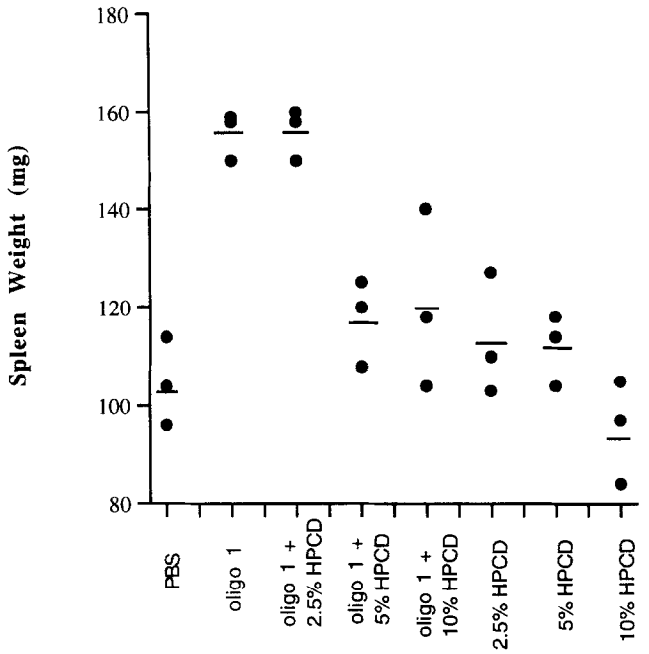

FIG. 4. Effect of HPCD on splenomegaly induced by oligo 1. Mice were injected with oligo 1, or HPCD, or oligo 1 complexed with HPCD. At 48 hr post-injection, mice were killed, and spleens were harvested and weighed. The bar represents the mean weight of each group. The results shown are representative of three experiments.

for immunoglobulin production, there was a significant increase in IgM production from mice injected with oligo 1 ($P < 0.01$) (Fig. 5). About a 3-fold increase in IgM production was observed compared with PBS-treated mice. When the oligonucleotide was injected along with HPCD, there was a decrease in IgM production compared with oligo 1-treated mice. The reduction was dependent on the concentration of HPCD. Five percent HPCD produced better immunomodulatory effects than other concentrations ($P < 0.01$ compared with the oligo 1-treated group). HPCD, injected alone, did not have any significant effects on cell cycling or IgM production. Similar results were observed with another cyclodextrin analog. HECD (data not shown). These results support the *in vitro* data suggesting that cyclodextrin analogs can modulate the immunostimulatory effect of oligonucleotides. The immunomodulatory effects of cyclodextrin analogs on oligonucleotide were not prolonged, however, and the suppression disappeared 96 hr after administration (Fig. 6). The mechanism of reversibility is not yet understood, but could be due to differential clearance. Since cyclodextrins are cleared out of the body within 24 hr of administration [16] while the oligonucleotides have a longer half-life in animals [21], this reversibility may be due to the dissociation of the oligonucleotide/cyclodextrin complex and/or a difference in the metabolism of the two compounds in the body.

To further confirm the immunosuppressive effects of cyclodextrin analogs on oligo 1, we carried out similar experiments with another oligonucleotide, oligo 2. Mice were injected with oligo 2 at 25 mg/kg and euthanized 24 hr later. Cell cycle analysis showed that injection of oligo 2 alone stimulated cell proliferation. When oligo 2 was complexed to 5% HPCD, the stimulation was reduced markedly to the same level as the vehicle alone (Table 1). Measurement of IgM antibodies showed that oligo 2 increased IgM production by approximately 10-fold ($P < 0.01$) (Fig. 5). In the presence of 5% HPCD, IgM production decreased significantly ($P < 0.01$).

TABLE 1. Cell cycle analysis

	Percentage of cells in:		
	G_0/G_1	G_2/S	% Change
A			
PBS	91.4 ± 0.1	8.6 ± 0.1	Control
2.5% HPCD	91.0 ± 0.3	9.0 ± 0.3	+4.6
5% HPCD	91.9 ± 0.9	8.1 ± 0.9	−5.8
10% HPCD	91.2 ± 0.5	8.8 ± 1.2	+2.3
B			
PBS	86.4 ± 0.1	13.6 ± 0.1	Control
5% HPCD	86.7 ± 0.8	13.3 ± 0.8	0
Oligo 1	82.0 ± 1.2	18.0 ± 1.2	+32
Oligo 1 + 2.5% HPCD	84.5 ± 1.7	15.5 ± 1.7	+14
Oligo 1+ 5% HPCD	85.7 ± 0.7	14.3 ± 0.7	+5
Oligo 1 + 10% HPCD	86.7 ± 2.3	13.3 ± 2.3	0
C			
PBS	77.7 ± 0.7	22.3 ± 0.7	Control
Oligo 2	68.5 ± 2.0	31.5 ± 2.0	+41
Oligo 2 + 5% HPCD	77.3 ± 0.5	22.7 ± 0.5	+1

Mice were injected with HPCD or oligonucleotides, or oligonucleotides complexed with HPCD. At 48 hr post-injection (in the case of sections A and B) or 24 hr (section C), mice were killed. Spleen lymphocytes were harvested, and cell cycle analysis was carried out as described in Materials and Methods. The results are presented as means ± SD of quadruplicate experiments. A, B, and C were separate experiments.

Besides single administration, cyclodextrin analogs also exert their immunomodulatory effects with multiple administrations of oligonucleotides. Oligo 2 was injected daily at a dose of 5 mg/kg for 7 days either alone or in complexation with 5% HPCD, and the mice were killed at day 8. Administration of the oligonucleotide alone resulted in a dramatic increase in spleen weight (more than 6-fold increase compared with mice in the control group), which was reduced markedly when the oligonucleotide was injected together with cyclodextrin analogs (36% decrease compared with the oligo 2-injected group, $P < 0.05$).

DISCUSSION

Phosphorothioate oligonucleotides are effective inhibitors of various genes in cell culture and in animals [1, 2]. The potential utility of antisense oligonucleotide therapy for treatment of viral infections and selected cancers, as well as various other parameters, depends on the outcome of toxicological and pharmacological studies. Relatively little is

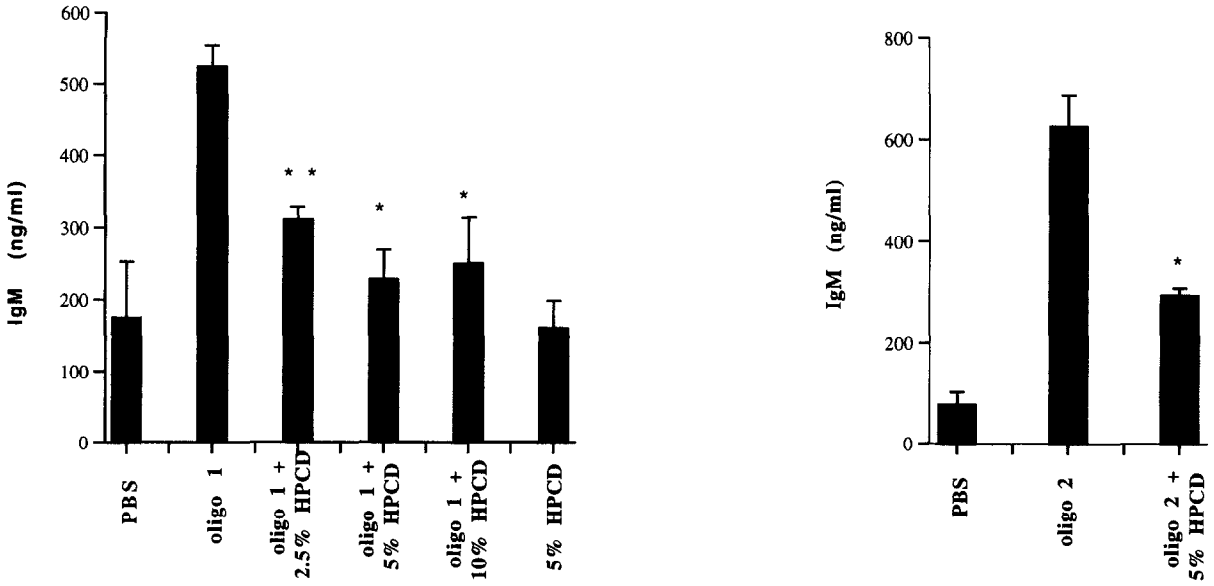

FIG. 5. IgM production in mice injected with oligonucleotide either alone or complexed with various-concentrations of HPCD. Values are means ± SD of quadruplicate experiments. Key: (*) $P < 0.01$ and (**) $P < 0.05$, compared with the oligonucleotide-injected group.

known about the side-effects of these oligonucleotides, especially on the immune system, but recent reports show that some phosphorothioate oligonucleotides can induce cell proliferation, splenomegaly, and antibody production (IgG and IgM) [6–10, 22]. Although the exact mechanism by which the oligonucleotides stimulate the immune system is still unclear, it seems to be dependent on particular sequences [6–9] and chemical modification of oligonucleotides [10].

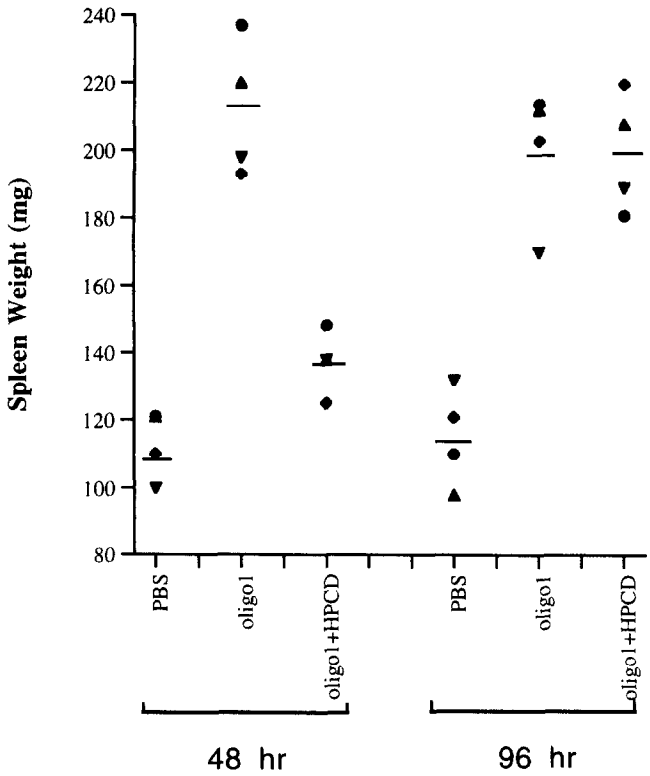

FIG. 6. Spleen weights of four animals 48 and 96 hr after injection of oligo 1 alone or complexed to 5% HPCD. The bar represents the mean weight of each group.

We have shown recently that cyclodextrin analogs can increase cellular uptake of oligonucleotides by 2- to 3-fold [19, 20]. In a continuing effort to develop cyclodextrin analogs as a delivery system for oligonucleotides, we assessed the effects of cyclodextrin analogs on oligonucleotide-induced immune stimulation. We used two oligonucleotides (oligo 1 and oligo 2) that cause immune stimulation [6, 9], and three cyclodextrin analogs (HPCD, HECD, and Encapsin) that increase cellular uptake of oligonucleotides [19].

We have shown that all three cyclodextrin analogs reduced the mitogenicity of oligo 1 and oligo 2 *in vitro*. The immunosuppressive effects of cyclodextrin analogs were also observed *in vivo*. Administration of oligo 1 or oligo 2 into mice resulted in splenomegaly and an increase in IgM production. Administration of the same oligonucleotides complexed to the cyclodextrin analogs (HPCD and HECD) resulted in a reduced spleen weight and a decrease in IgM production after 48-hr administration. The suppression was dependent on the concentration of cyclodextrin analogs. A concentration of at least 5% was necessary to achieve significant immunosuppressive effects. At this concentration, the molar ratio of the cyclodextrin/oligonucleotide was about 80, suggesting that many cyclodextrin molecules are needed to interact with one molecule of oligonucleotide. This result supports those of previous studies in which a concentration of cyclodextrin between 0.5 and 40%, and a large excess of cyclodextrin analogs over the drug were used [11–16, 23]. Injection of cyclodextrin analogs alone had no significant effect on immune stimulation. In addition, no significant immunosuppressive effects were observed when

cyclodextrin analogs and oligonucleotides were injected simultaneously but at opposite sites or at the same site but 24 hr apart. These experiments indicate that the cyclodextrin analogs by themselves do not have immunosuppressive or cytotoxic effects on newly activated cells. Furthermore, it seems that the interaction between cyclodextrin analogs and oligonucleotides is required for the immune modulatory effect to occur.

The mechanism by which cyclodextrin analogs suppress the oligonucleotide-induced immune stimulation is not fully understood. Phosphorothioate oligonucleotides mainly exert their immune stimulation effects through B cells; there are some indications that T cells, natural killer cells, and macrophages are also involved in the process [8, 9]. It has been demonstrated that cell uptake of oligonucleotide is required for the immune stimulation to occur [9]. It is possible that lymphocyte activation arises from discrete protein(s) interaction on the cell membrane or following entry of the oligonucleotide into the cell. Phosphorothioate oligonucleotides have been shown to bind to proteins in a non-specific manner [2]. Cyclodextrin can interact with a variety of molecules through its unique hydrophobic cavity [24]. It is possible that an interaction of this type with oligonucleotides reduces the non-specific binding of oligonucleotides with proteins, thereby reducing their immune stimulation. Since the association between cyclodextrin and oligonucleotide is not strong, the dissociation of oligonucleotide from cyclodextrin upon administration *in vivo*, and a rapid clearance of cyclodextrin in the body versus a much longer half-life of oligonucleotide may account for the phenomenon of reversibility. An alternative mechanism is that cyclodextrin analogs change the biodistribution of the oligonucleotide and its accumulation in tissues. This seems unlikely, however, since preliminary pharmacokinetic studies using radiolabeled oligonucleotide indicate that the tissue distribution of the cyclodextrin/oligonucleotide is similar to that of free oligonucleotide (Agrawal *et al.*, unpublished data). The immunosuppressive effect of cyclodextrin analogs may not be due to their cytotoxic effects, since injection of cyclodextrin analogs alone had no significant effect on immune stimulation, or to the cytotoxic effects to newly activated cells, since no significant immunosuppressive effects were observed when cyclodextrin analogs and oligonucleotides were injected simultaneously but at opposite sites or at the same site but 24 hr apart. Cyclodextrin analogs do not inhibit hybridization of the oligonucleotide to the target since the T_m of duplex formation with RNA is similar for free and cyclodextrin-bound oligonucleotide. Besides reducing the immunostimulatory effect of oligonucleotides, we have found that cyclodextrin analogs can reduce some other undesirable side-effects of oligonucleotides such as reduction of platelet counts, a phenomenon that has been observed with phosphorothioate oligonucleotides [7], and can delay the degradation of oligonucleotides for up to 24 hr *in vivo* (Agrawal *et al.*, unpublished data).

Besides oligonucleotides, we observed that cyclodextrin analogs also have immunosuppressive effects on other immunostimulatory compounds (including lipopolysaccharide and pokeweed mitogen) *in vitro* (Zhao *et al.*, unpublished data). The effect *in vivo* will depend, however, on the interaction of cyclodextrin analogs and the guest compounds and their pharmacokinetics in the body. Studies are underway to evaluate the effect of cyclodextrin analogs on other immunostimulatory compounds.

It will be of interest to define precisely the mechanism of action of cyclodextrin analogs so that better formulations of oligonucleotides/cyclodextrin analogs can be developed. In summary, cyclodextrin analogs are attractive molecules to use as carriers for oligonucleotides. They can increase cellular uptake of oligonucleotides and improve several side-effects of oligonucleotides, such as immunostimulatory effects.

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