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Effect of Different Chemically Modified Oligodeoxynucleotides on Immune Stimulation

*Qiuyan Zhao, Jamal Temsamani, Patricia L. Iadarola, Zhiwei Jiang and Sudhir Agrawal**
HYBRIDON, INC., WORCESTER, MA 01605, U.S.A.

ABSTRACT. Based on previous studies that certain oligonucleotides can stimulate cell proliferation and immunoglobulin production, this study was carried out to establish the relationship between the stimulatory effect and the chemical modification of the oligonucleotide. First, the effects of oligonucleotide and analogs on immune stimulation were studied *in vitro* using murine splenic lymphocytes. Our results show that cell proliferation and immunoglobulin production (IgG and IgM) depend on the sequence and the chemical modification of the oligonucleotide. Phosphorothioate oligodeoxynucleotides displayed a greater stimulatory effect than partially modified phosphorothioate oligonucleotides. Second, we studied the effects of these chemically modified olionucleotides after injection in mice. Massive splenomegaly and stimulation of cell proliferation were observed with some phosphorothioate oligonucleotides. These effect were minimized markedly by chimeric and hybrid oligonucleotides. We also demonstrate that *in vitro* the effects of oligonucleotides on murine lymphocytes were unaffected by T cell depletion, suggesting that oligonucleotides exert their effects mainly on the B cells. BIOCHEM PHARMACOL 51;2:173–182, 1996.

KEY WORDS. oligonucleotide; chemical modification; cell proliferation; immune stimulation

Antisense oligonucleotide technology presents an exciting new therapy for many diseases, including viral infections and cancer. The oligonucleotide binds to its target RNA by Watson–Crick base-pairing. In principle, an oligonucleotide could be designed to target any single gene within the human genome. Therefore, the antisense approach is currently the only known strategy that has broad potential for precise and effective modulation of the expression of specific genes in a disease situation. The field has progressed enormously over the past decade, and today there are numerous reports in the literature documenting the effectiveness of various antisense oligonucleotides [1] in modulating the expression of viral genes or of oncogenes in *in vitro* and *in vivo* situations [2–5]. Because of their favorable results *in vitro*, the clinical relevance of antisense oligonucleotides is demonstrated by numerous clinical trials currently in progress or proposed [6–8]. The practical application of this exciting new approach will depend upon the outcome of toxicological and pharmacological studies. One important issue is the effect of these oligonucleotides on the immune system.

Large DNA molecules, strands of dC and dG, and double-stranded RNA have been shown to induce significant cell proliferation and antibody response when administered to mice [9–11]. Oligonucleotides are small synthetic DNA molecules, resembling single-stranded DNA. The presence of chemically modified linkages (i.e. phosphorothioate), unusual conformations, or structural determinants that are rare in mammalian cells may induce an immunostimulatory effect.

In our continued interest in developing antisense oligonucleotides as therapeutic agents, we studied the effects of oligonucleotides and analogs on immune stimulation. We assessed the effects of different chemical modifications, sequences, and lengths of oligonucleotides on cell proliferation and antibody production by murine cells, both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Synthesis, Deprotection and Purification of Oligonucleotides

OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES. Oligodeoxynucleotide phosphorothioates were synthesized using an automated DNA synthesizer (model 8700, Biosearch, Bedford, MA) using the β-cyanoethyl phosphoramidite approach on a 10 μM scale. To generate the phosphorothioate linkages, oxidation of the intermediate phosphite linkage obtained after each coupling was carried out using 3*H*-1,2-benzodithiole-3*H*-one-1,1-dioxide [12]. Oligodeoxynucleotides containing phosphodiester linkages were synthesized by using the same protocol as above, except to generate phosphodiester linkages; oxidation of the intermediate phosphite linkage obtained after each coupling was carried out with standard iodine reagent.

* Corresponding author: Dr. Sudhir Agrawal, Hybridon, Inc., One Innovation Dr., Worcester, MA 01605. Tel. (508) 752-7000; FAX (508) 751-7692.
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CHIMERIC OLIGONUCLEOTIDES. Synthesis of chimeric oligonucleotides (GEM 91†-C and Rev-C) was carried out on a 10-μM scale using the same instrument as above. Segments of chimeric oligonucleotide containing methylphosphonate linkages were assembled using nucleoside methylphosphonamidites (Glen Research, Sterling, VA) followed by oxidation with 0.1 M iodine in tetrahydrofuran:2,6-lutidine:water, 75:25:0.25). The segment of chimeric oligonucleotide containing phosphorothioate linkage was assembled using the same procedure as described above for the oligonucleotide phosphorothioate. The deprotection of chimeric oligonucleotide was carried out in two steps. First, the CPG-bound chimeric oligonucleotide was treated with concentrated ammonium hydroxide for 1 hr at room temperature, and the supernate was removed and evaporated to obtain a pale yellow residue. Then the dried residue was treated with a mixture of ethylenediamine:ethanol (1:1, v/v) for 6 hr and was dried again under reduced pressure.

HYBRID OLIGONUCLEOTIDE. Hybrid oligonucleotides (GEM 91-H and Rev-H) were synthesized on a 10-μM scale using the same instrument as above. The segment containing 2′-*O*-methylribonucleotide was synthesized using 2′-*O*-methylribonucleoside β-cyanoethylphosphoramidites followed by oxidation with 3*H*-1,2-benzodithiole-3*H*-one-1,1-dioxide [12]. The segment containing phosphorothioate linkages was synthesized by the same procedure as described above for oligodeoxynucleotide phosphorothioate.

The deprotection (except for the chimeric oligonucleotides) and purification of all the oligonucleotides were carried out by the same procedure as described earlier [13]. Purity of the oligonucleotides was checked on a 20% polyacrylamide gel containing 7 M urea, and contained more than 90% of the required length.

The sequences of oligonucleotides used in the present studies are: GEM 91: 5′-CTCTCGCACCCATCTCTCTCCTTCT-3′ [25-mer]; Rev: 5′-TCGTCGCTGTCTCCGCTTCTTCTTGCC-3′ [27-mer]; p53: 5′-CCCTGCTCCCCCCTGGCTCC-3′ [20-mer]; oligo 6-mer: 5′-CGCGCG [6-mer]; and oligo 10-mer: 5′-GACGATCGTC-3′ [10-mer].

Cell Proliferation

IN VITRO STUDIES. Spleen was taken from a male CD1 mouse (4–5 weeks, 20–22 g, Charles River, Wilmington, MA). Single cell suspensions were prepared by gently mincing with the frosted ends of glass slides. Cells were then cultured in RPMI complete medium [RPMI medium supplemented with 10% fetal bovine serum (FBS) (heat-inactivated at 56° for 30 min for studies with phosphorothioate, chimeric, and hybrid oligonucleotides and at 65° for studies with oligonucleotides containing phosphodiester segments to minimize degradation of oligonucleotides), 50 μM 2-mercaptoethanol (2-ME), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine]. 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 or mitogens (ConA, PWM, or LPS; 10 μg/mL) were added to the cell culture in 10 μL of TE buffer [10 mM Tris–HCl (pH 7.5), 1 mM EDTA]. The cells were then set to culture at 37°. After 44 hr, 1 μCi [^{3}H]thymidine (Amersham, Arlington Heights, IL) was added to the culture in 20 μL of RPMI medium, and the cells were pulse-labeled for another 4 hr. Then the cells were harvested by an automatic cell harvester (Skatron, Sterling, VA), and the filters were counted by a scintillation counter. The experiments were done in triplicate.

In the case of cell cycle analysis, the cells were cultured for 4 hr and then washed with FACS buffer [1× Hanks' Balanced Salt Solution (HBSS) supplemented with 1% BSA and 0.1% sodium azide]. The cells were fixed with 70% cold alcohol and then put on ice for 30 min. After fixation, cells were washed, then resuspended with 200 μL PBS, and treated with 50 μL of RNase (10 mg/mL, DNase free) at 37° for 30 min. Propidium iodide (50 μg/mL) was added to cells before flow cytometry analysis. Flow cytometric data on 10,000 viable cells were acquired in a histogram on an Epics XL flow cytometer (Coulter, Hialeah, FL), and data were analyzed by Epics XL version 1.5 software and multicycle software (Phoenix Flow Systems, San Diego, CA) after gating on living cells by forward scatter versus side scatter and excluding doublets. The experiments were done in duplicate.

Depletion of T Cells

A spleen was taken from a CD1 mouse, and single cell suspensions were prepared and resuspended in RPMI complete medium at a density of 10^7 cells/mL in a volume of 1 mL. Cells were incubated with rat anti-mouse Thy 1.2 (1:100) (Gibco BRL, Gaithersburg, MD) at 4° for 30 min. The cells were then washed, resuspended in RPMI medium, and incubated with Low-Tox-M Rabbit complement (1:100) (Cedarlane, Ontario, Canada) at 37° for 30 min. After passing through lymphocyte M (Cedarlane) to remove destroyed T cells, cells were washed thoroughly and resuspended in RPMI complete medium, and cell proliferation studies were performed as described above.

Antibody Production

IN VITRO STUDIES. Murine splenocytes (10^6 cells/mL) were cultured with oligonucleotides or medium alone for 9 days in 1-mL cultures. Cell culture supernatants were then collected and assayed for IgG and IgM levels using a standard ELISA assay. Briefly, ELISA plates (96 wells) were coated with goat anti-mouse IgG or IgM (5 μg/mL) diluted in PBS supplemented with 0.05% sodium azide (pH 9.6) overnight at 4°, washed three times with PBS-T buffer (PBS supplemented with 0.05% Tween 20 and 0.25% BSA), and incubated with cell culture supernatants at 37° for 2 hr. A standard of mouse IgG and IgM (1 mg/mL) was diluted with BPS-T buffer to provide a standard curve between 0 and 800 ng/mL. The plates

† Abbreviations: ConA, concanavalin A; CPG, controlled pore glass; GEM 91, gene expression modulator 91; HIV-1, human immunodeficiency virus type-1; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; PBMCs, primary blood mononuclear cells; and PWM, pokeweed mitogen.

were then washed three times with PBS-T buffer and incubated with goat anti-mouse IgG conjugated to alkaline phosphatase diluted 1:1000 with PBS-T buffer, and incubated at 37° for 2 hr. After three washes with PBS-T buffer, phosphatase substrate (*p*-nitrophenylphosphate 1 mg/mL) in diethanolamine (75 μL) was added to the plates, which were kept for 1 hr at room temperature. The colorimetric reaction was stopped by addition of 25 μL of 0.5 M sodium hydroxide. The optical density (405 nm) was measured using the reader Ceres 900 HDI (Bio-Tek Instruments, Winooski, VT). IgG and IgM levels were calculated based on the standard curve. The experiments were done in quadruplicate.

IN VIVO STUDIES. Male CD1 mice (4–5 weeks, 20–22 g, Charles River, Wilmington, MA) 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). The animals were injected intraperitoneally with 1 mg of oligonucleotide in 0.25 mL of PBS. Three animals were used for each oligonucleotide. Mice were killed 48 hr later, spleens were removed, and single cell suspension cell cultures were prepared and set up in culture at a density of 10^6 cells/ml. After 24 hr of culture, the supernatants were harvested and assayed for IgG and IgM as described above. The experiments were done in quadruplicate.

RESULTS

In Vitro *Studies*

In all the experiments carried out *in vitro*, we used three mitogens as control for cell proliferation: Con A, a mitogen specific for T cells; PWM, a mitogen specific for both T and B lymphocytes; and LPS, a mitogen specific for B lymphocytes.

To assess the *in vitro* effect of oligonucleotides on murine cell proliferation, we incubated murine splenocytes with different concentrations of oligonucleotides (0.1, 1, 10, and 100 μg/mL) for 48 hr as described in Materials and Methods. The cells were then pulse-labeled with [^{3}H]thymidine for 4 hr, and the incorporation of thymidine was measured. We used five oligonucleotides of varying length and sequence. The first one, Rev, a 27-mer phosphorothioate oligonucleotide complementary to the *rev* region of HIV-1, has been shown to stimulate the proliferation of spleen cells both *in vitro* and *in vivo* [14]. The second, p53, is a 20-mer phosphorothioate oligonucleotide that has been shown by the same investigators to have no effect on cell proliferation. The phosphorothioate oligonucleotides oligo 6-mer and oligo 10-mer that contain palindromic sequences have been shown to induce interferon production and natural killer cell activity [15]. The last oligonucleotide, GEM 91, is a 25-mer phosphorothioate oligonucleotide complementary to the *gag* region of HIV [16, 17]. As expected, Fig. 1 shows that the mitogens ConA, PWM, and LPS induced cell proliferation; P53 oligonucleotide did not induce any significant cell proliferation at any of the concentrations studied. Rev oligonucleotide stimulated the proliferation of murine splenocytes in a concentration-dependent manner. At 100 μg/mL, the thymidine uptake increased by more

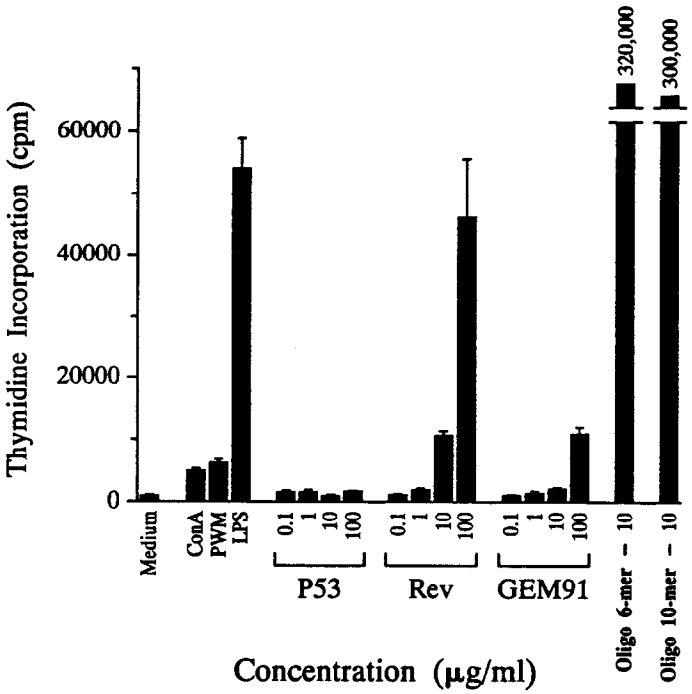

FIG. 1. Effects of oligonucleotides on *in vitro* murine splenocyte proliferation. Cells were incubated for 48 hr with different concentrations of oligonucleotides and then were pulse-labeled with [^{3}H]thymidine. Ten micrograms per milliliter of p53, Rev, GEM 91, oligo 6-mer, and oligo 10-mer corresponds to a concentration of 1.5, 1.14, 1.2, 5.1, and 3 μM, respectively. In the case of oligo 6-mer and oligo 10-mer, the bars have been reduced to fit within the graph format. Results are means ± SD of triplicate cultures and are representative of three experiments.

than 50-fold compared with medium alone, and confirms previously reported results [14]. The oligonucleotides oligo 6-mer and oligo 10-mer, which contain palindromic CG sequences, were also used in the present study to compare the level of cell proliferation with that of GEM 91, Rev and p53 oligos. Both oligo 6-mer and oligo 10-mer showed markedly high levels of cell proliferation compared with the other three oligonucleotides, even at a concentration of 10 μg/mL. GEM 91 also induced stimulation of lymphocyte proliferation; however, it was much less than that induced by Rev, oligo 6-mer, or oligo 10-mer. At 100 μg/mL, GEM 91 induced thymidine uptake by approximately 10-fold compared with medium alone. The effects of these oligomers were not species-specific since similar results were obtained when the experiment was carried out with human PBMCs or with monkey spleen mononuclear cells (data not shown). These results seem to indicate that the stimulation of murine splenocytes is concentration dependent and relatively specific to oligonucleotide sequence.

EFFECT OF LENGTH. To study the effect of the length of the phosphorothioate oligonucleotides on murine cell proliferation, different lengths (16-, 18-, 20-, and 23-, and 25-mers) of the phosphorothioate oligonucleotide GEM 91 were synthesized and assayed for *in vitro* proliferation of murine splenocytes. The length of GEM 91 was reduced from either the 5′-end or the 3′-end (Fig. 2). The oligonucleotides were incu-

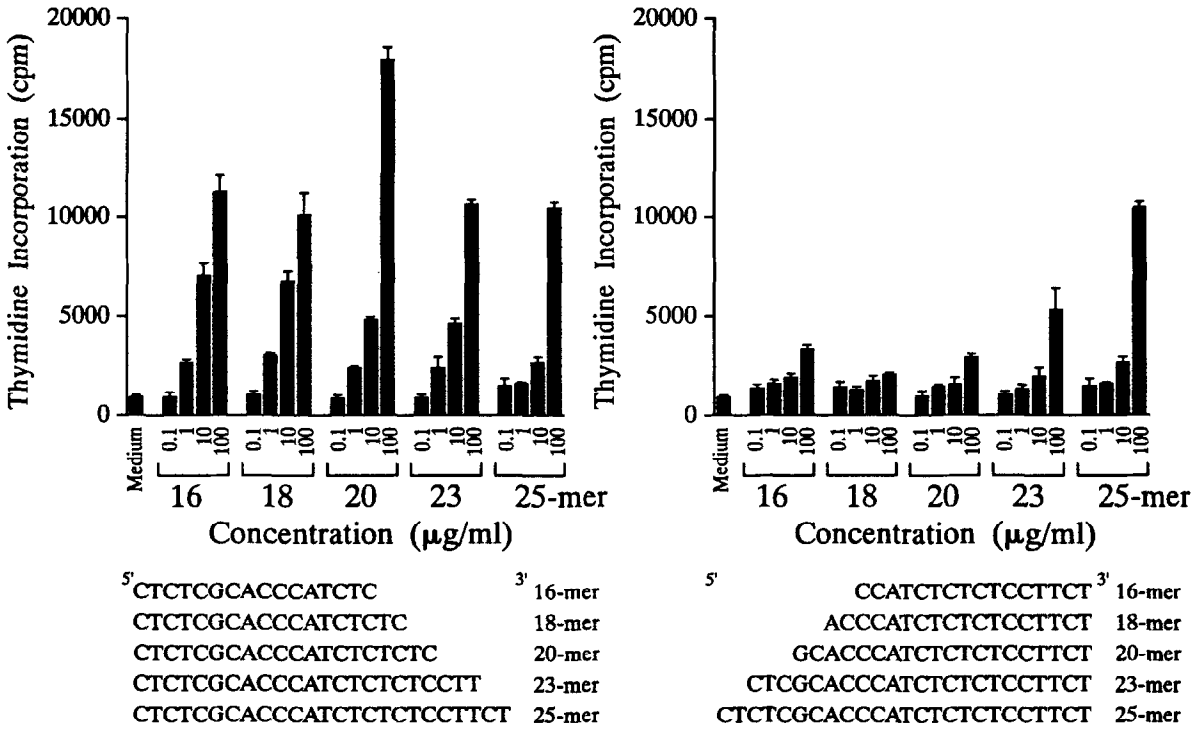

FIG. 2. Effect of the length of phosphorothioate oligonucleotides on murine splenocyte proliferation. GEM 91 length was reduced either from the 5′- or the 3′-end. Cells were incubated with these different oligomers and pulse-labeled with [^{3}H]thymidine. The final concentration of 10 μg/mL corresponds to 1.9 to 1.2 μM for a 16- to 25-mer. Bars represent the SD of triplicate cultures.

bated with the murine spleen cells at different concentrations (0.1, 1, 10, and 100 μg/mL). Figure 2 shows that when the length of the oligonucleotide was reduced from the 5′-end, the cell stimulation was length dependent. The 16-mer, 18-mer and 20-mer did not induce any significant increase in cell proliferation, but when the length increased to 23 or to the initial length (25-mer), the uptake of thymidine increased. There was no significant increase of proliferation at 0.1, 1, or 10 μg/mL for the 5′-end reduced oligonucleotides. Surprisingly, when the length of the oligonucleotide was reduced from the 3′-end, the results were different. All the different lengths of the oligonucleotide showed approximately similar levels of cell proliferation as GEM 91. This result suggests that the stimulation of murine splenocytes is not length dependent but rather sequence or structure dependent. It is possible that when the length of the oligomer was reduced from the 5′-end, an important structure or motif for inducing cell proliferation was removed.

EFFECT OF CHEMICAL MODIFICATIONS. To study the effect of chemical modifications on the stimulation of cell proliferation and immunoglobulin production, GEM 91-C, GEM 91-H, and GEM 91-L oligonucleotides were synthesized (Fig. 3). GEM 91-C and GEM 91-H have the same sequence and length as GEM 91; however, GEM 91-C (*chimeric*) has four methylphosphonate linkages at each end of the oligonucleotide, and GEM 91-H (*hybrid*) has four 2′-O-methylribonucleotide residues at each end. GEM 91-L (*loop*) is all phosphorothioate but contains a stem loop at the 3′-end [18]. Figure 3 shows that GEM 91-L oligonucleotide induced a level of cell proliferation similar to that induced by GEM 91. Interestingly, GEM 91-C and GEM 91-H oligomers did not induce significant cell proliferation at any of the concentrations studied. We also measured immunoglobulin production. Murine splenocytes were incubated with GEM 91, GEM 91-C, GEM 91-H, and GEM 91-L oligonucleotides. The IgG and IgM production was measured by the ELISA test as described in Materials and Methods. We measured IgG and IgM production after 2, 5, 8, and 10 days of incubation; however, the difference in IgG and IgM production was detectable only after 8 days of incubation (data not shown). We, therefore, carried out the following experiment for 9 days. Figure 4 shows that antibody production was concentration dependent. GEM 91 and GEM 91-L oligonucleotides induced similar levels of IgG and IgM antibodies (Fig. 4). Interestingly, the stimulation of antibody production by GEM 91-C and GEM 91-H oligomers was much lower.

To confirm that the effect of chemical modifications on cell stimulation and immunoglobulin production are not restricted to GEM 91, the same modifications were introduced in the Rev oligonucleotide (Fig. 5). Figure 5 shows clearly that Rev-C (*chimeric*) and Rev-H (*hybrid*) markedly reduced the cell proliferation. These results suggest that the stimulation of murine splenocytes is dependent on the chemical modification of the oligonucleotide. To explore this possibility further, we made GEM 91 with different numbers of phosphodiester linkages. Six oligonucleotides were synthesized and assayed for their effects on the proliferation of murine spleen cells (Fig. 6). In this case, the experiment was carried out in heat-inacti-

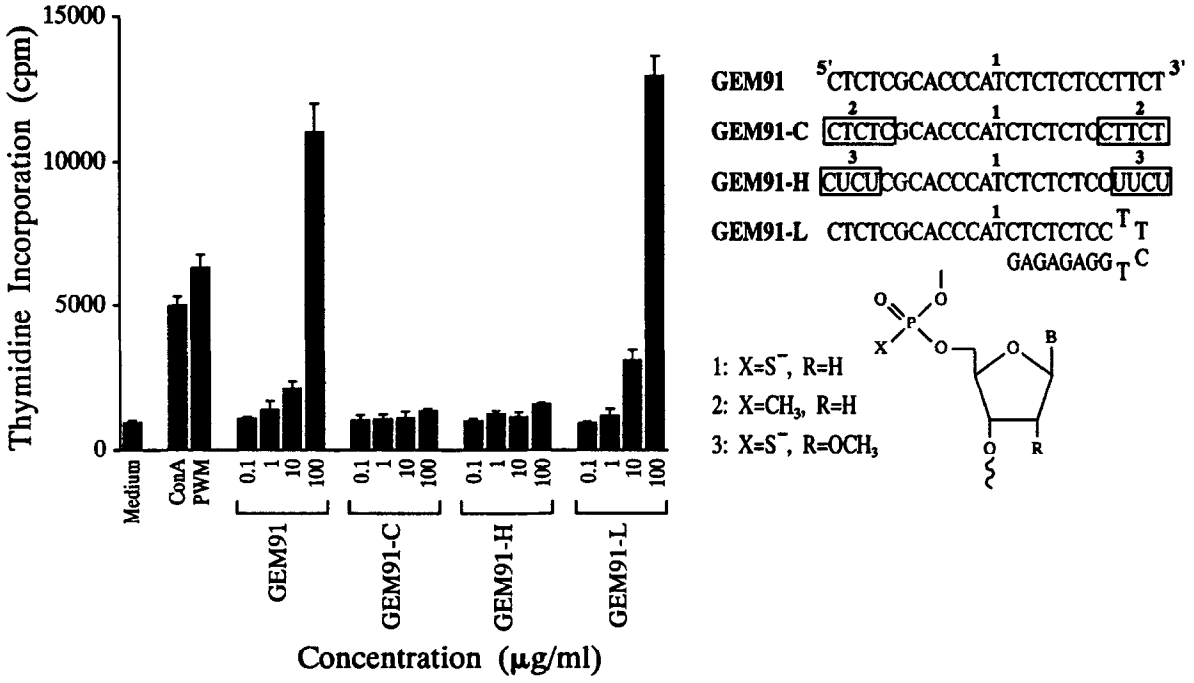

FIG. 3. Effects of GEM 91 and its analogs on murine splenocyte proliferation. The chemical modifications of GEM 91 are indicated. The numbers 1, 2, and 3 represent the different linkages. Bars represent the SD of triplicate cultures.

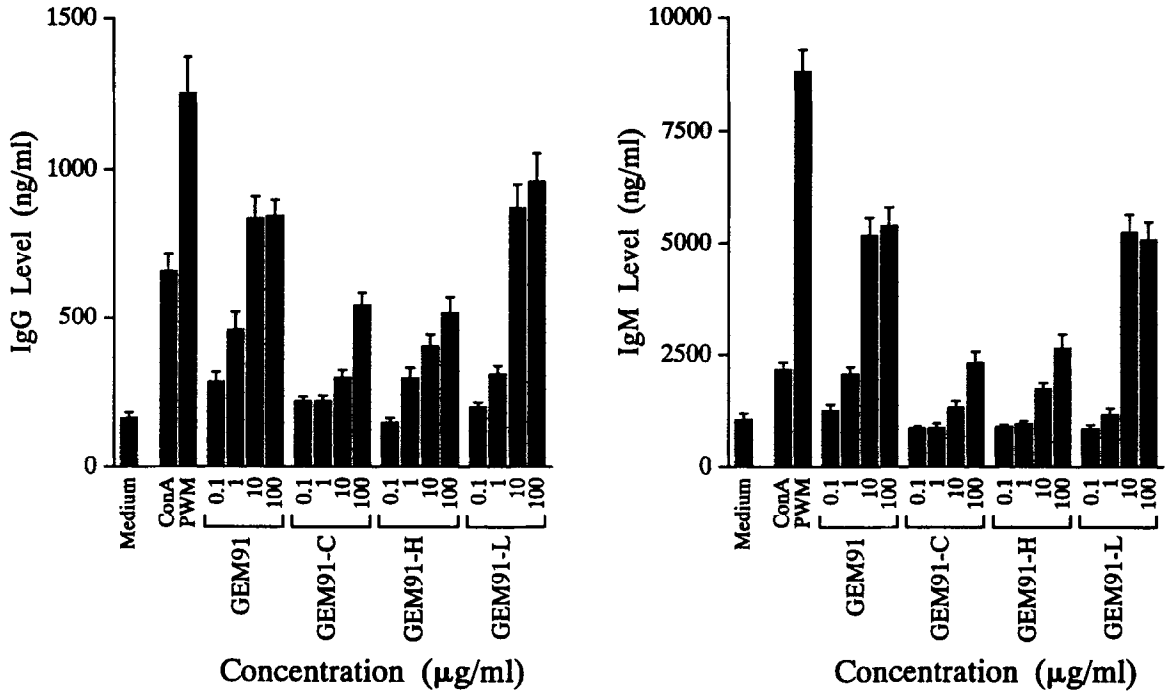

FIG. 4. Effects of different modified oligonucleotides on IgG and IgM production by murine splenocytes. Oligonucleotides were incubated with murine splenocytes for 9 days. The supernatants of cell cultures were measured for IgG and IgM. The chemical modifications of the oligonucleotides are shown in Fig. 3. Bars represent the SD of triplicate cultures.

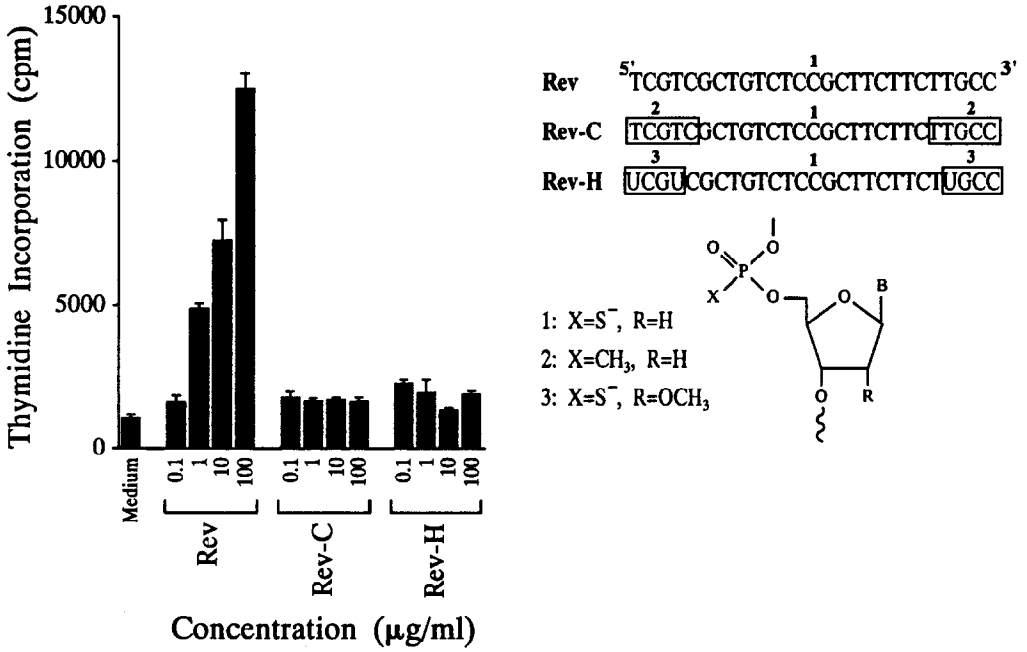

FIG. 5. Effects of Rev and its analogs on murine splenocyte proliferation. The chemical modifications of Rev oligonucleotide are indicated. The numbers 1, 2, and 3 represent the different linkages. Bars represent the SD of triplicate cultures.

vated medium at 65° for 30 min to avoid any degradation of phosphodiester linkages. Figure 6 shows that the unmodified or partially unmodified oligonucleotides did not induce significant cell proliferation. We also attempted to assess the effects of unmodified and partially unmodified oligonucleotides on antibody production, but unfortunately the cells did not grow efficiently for 9 days in heat-inactivated medium.

In Vivo *Studies*

It was of interest to test whether these effects of different oligonucleotides were applicable *in vivo*. CD1 mice were injected intraperitoneally with a dose of 50 mg/kg of oligonucleotide per body weight; 48 hr later, the animals were euthanized, and the spleens were removed and weighed. The spleens of animals treated with Rev oligonucleotide were approximately twice in size (100% increase) as those of mice injected with vehicle alone (PBS) (Fig. 7), whereas GEM 91 and GEM 91-L induced only a 15% increase in spleen. Interestingly, the modified analogs GEM 91-C, GEM 91-H, Rev-C, and Rev-H did not induce any significant increase in spleen weight. To further investigate the splenomegaly observed, we measured the proliferation of spleen cells by cell cycle analysis. Table 1 shows that after staining fixed spleen cells with propidium iodide, there was a marked increase in the percentage of cells in the S/G_2 phases from mice treated with GEM 91, GEM 91-L, and Rev oligonucleotides compared with untreated mice. The phosphorothioate oligonucleotide Rev induced about a 30% increase in the percentage of cells in S/G_2 phases of the cell cycle, compared with the vehicle alone. GEM 91 and GEM 91-L induced only 13% increase. In the case of the modified analogs (GEM 91-C, GEM 91-H, Rev-C, and Rev-H), there was no significant increase in cycling cells, confirming the results observed *in vitro*. Similar results were obtained when cell proliferation was measured by [^{3}H]thymidine uptake. Similarly, when the spleen cells were measured for immunoglobulin production, there was a marked increase in production of both IgG and IgM from mice injected with Rev oligonucleotide (Fig. 8). About 3-fold increase in IgG and IgM production was observed compared with results from the vehicle alone. In the case of GEM 91, there was no significant increase in antibody production (data not shown). The modified analogs, Rev-C and Rev-H, minimized the effect observed with the Rev oligonucleotide. To rule out that the immunostimulatory effect observed with intraperitoneal injection is not due to a difference in accumulation of Rev, Rev-C, and Rev-H in spleen, we carried out a similar experiment using intravenous injection. In our previous pharmacokinetic studies ([19, 20], and unpublished results), we have shown that phosphorothioate, chimeric, and hybrid oligonucleotides display similar accumulations in spleen and other tissues after intravenous injection. Similar results were observed when the experiment was carried out with intravenous injection. These results suggest that phosphorothioate oligonucleotides, not the modified analogs, activated spleen cell proliferation and induced IgG and IgM production.

Effect of T Cell Depletion on Cell Proliferation

To test the effect of T cells on cell proliferation, we carried out *in vitro* immunodepletion experiments by incubating murine spleen cells with a monoclonal antibody specific for T cells,

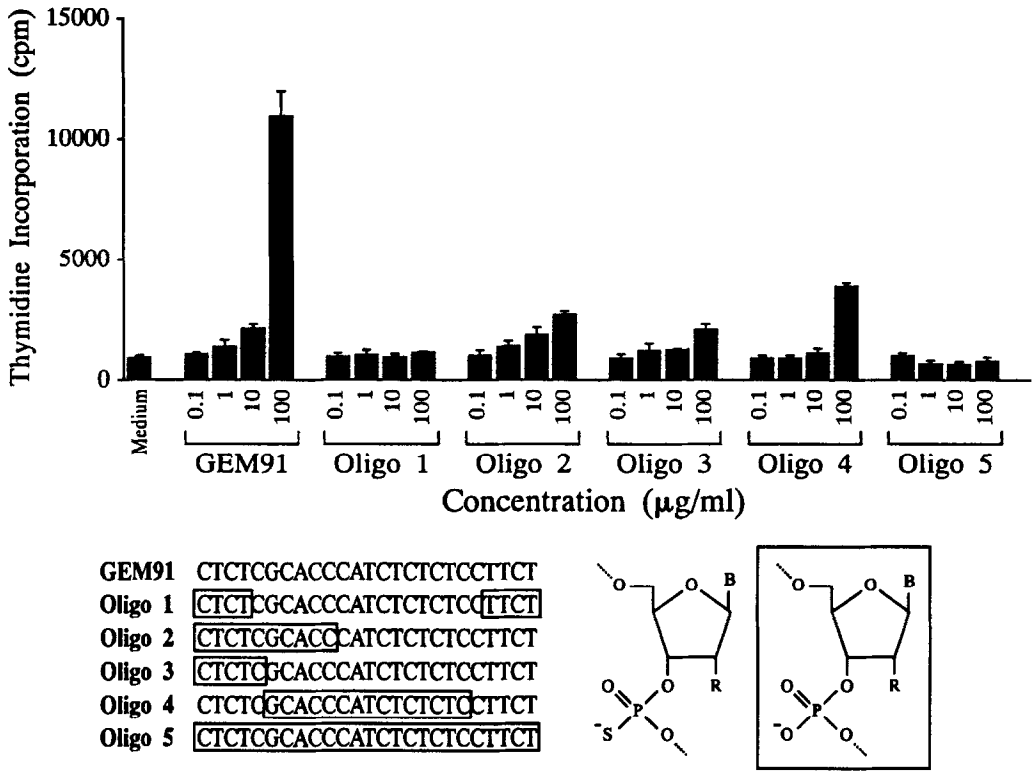

FIG. 6. Effects of unmodified and phosphorothioate-modified oligonucleotides on murine splenocyte proliferation. The nucleotides enclosed in a box are of phosphodiester linkage, while the others are of phosphorothioate linkage. Results are means ± SD of triplicate cultures and are representative of three experiments.

followed by the addition of rabbit complement. The cells were then incubated with various concentrations of the GEM 91 oligonucleotide. Figure 9 shows that the stimulation of the mitogen ConA (specific for T cells) was approximately the same as the medium, indicating that the depletion of T cells was complete. GEM 91 induced cell proliferation in both the absence and presence of T cells, which suggests that the stimulation is independent of T cells. In the T-depleted cells, there was more stimulation with GEM 91 because the same number of cells was used in both depleted and non-depleted samples.

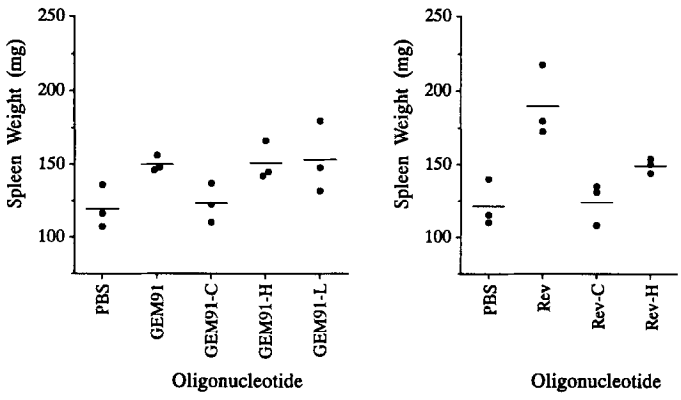

FIG. 7. Spleen weights of three animals after injection of PBS alone or different oligonucleotides. CDI mice were injected i.p. with a dose of 50 mg/kg of oligonucleotide per body weight; 48 hr later, the animals were euthanized, and the spleens were removed and weighed. The bar indicates the mean weight of each group.

DISCUSSION

Antisense oligonucleotides are short sequences of DNA that form specific hydrogen bonds with complementary target mRNA sequences allowing modulation of specific foreign or cellular gene expression. They are widely used as research tools for inhibiting gene expression and are under investigation for possible use as therapeutic agents. The potential utility of antisense oligonucleotide therapy for treatment of viral infections and selected cancers will depend on the outcome of toxicological and pharmacological studies. Relatively little is known about the *in vivo* side-effects of these oligonucleotides on different tissues, especially on the immune system. GEM 91 and Rev are phosphorothioate oligonucleotides complementary to *gag* and *rev* regions of HIV-1 and have been shown to inhibit HIV-1 replication [16, 17, 21]. Rev oligonucleotide has been shown to have an immune stimulatory effect both *in vitro* and *in vivo* [14]. In this report, we compared the immune effects of GEM 91 and Rev oligonucleotides. We also made various analogs of GEM 91 and Rev oligonucleotides and studied the impact of these modifications on cell proliferation and antibody production both *in vitro* and *in vivo*. Our results seem to indicate that immune stimulation depends on various factors. We show that oligonucleotides can induce cell prolifer-

TABLE 1. Cell Cycle Analysis

	Percentage of cells in:			Percentage of cells in:	
	G_1	S/G_2		G_1	S/G_2
PBS	88.3 ± 0.25	11.7 ± 0.20	PBS	85.4 ± 0.36	14.6 ± 0.32
GEM 91	86.9 ± 0.39	13.1 ± 0.38	Rev	81.3 ± 0.49	18.7 ± 0.45
GEM 91-C	88.4 ± 0.71	11.3 ± 0.29	Rev-C	85.9 ± 1.90	14.1 ± 1.90
GEM 91-H	88.3 ± 0.21	11.8 ± 0.21	Rev-H	85.7 ± 0.46	14.3 ± 0.46
GEM 91-L	87.7 ± 2.59	12.7 ± 1.96			

Cells from mice injected with oligonucleotide or vehicle alone (PBS) were cultured, and then cell cycle analysis was carried out as described in Materials and Methods. The experiments with GEM 91 and Rev oligonucleotides are two separate experiments. The results are means ± SD of a quadruplicate experiment.

ation and antibody production both *in vitro* and *in vivo*; yet these effects do not appear to be a general property of oligodeoxynucleotides but rather relatively specific to certain sequences or structures. For example, Rev, oligo 6-mer, and oligo 10-mer induced a marked cell proliferation while GEM 91 induced only a modest response. McIntyre *et al.* [22] showed that a 24-mer phosphorothioate oligonucleotide (p65 sense) induced splenic cell proliferation while the complementary 24-mer phosphorothioate oligonucleotide (p65 antisense) did not. This observation indicates that the stimulatory effects are dependent upon particular sequences of the oligonucleotide but independent of whether the oligonucleotide is antisense, sense, or scrambled with respect to their respective target genes. Certain sequence motifs or structures of oligonucleotides may play important roles in causing stimulation of murine cells. Kuramoto and his coworkers [15] found that the presence of particular palindromic sequences including the 5′-CG-3′ motif(s) is critical for oligonucleotides in inducing natural killer cell activation and interferon production. More recently, Krieg *et al.* [23] reported that optimal B cell activation requires a DNA motif in which an unmethylated CpG dinucleotide is flanked by two 5′ purines and two 3′ pyrimidines. These findings may explain the significant level of cell proliferation observed with oligo 6-mer, 10-mer, and Rev oligonucleotide, all of which contain palindrome sequences of the CG motif. Also they seem to corroborate with our own experiment in which the length of GEM 91 was reduced from either the 5′-end or the 3′-end. GEM 91 contains one CpG towards the 5′-end, and when the length of the oligonucleotide was reduced from this end, the stimulation of murine spleen cells was reduced markedly. Reducing the length of the oligonucleotide from the 3′-end did not seem to affect the levels of cell proliferation. Our findings strongly suggest that chemical modifications play an important role in the stimulatory effects. The degree of substitution with thioate linkages in the oligonucleotide can influence the stimulatory activity of the oligonucleotides. Oligonucleotides that contain fewer thioate groups, such as GEM 91-C, and partially modified oligonucleotides induced less immune stimulatory effects both *in vitro* and *in vivo*. These findings were not confined to the particular GEM 91 sequence, since the same modifications in the Rev sequence (Rev-C and Rev-H) markedly reduced the

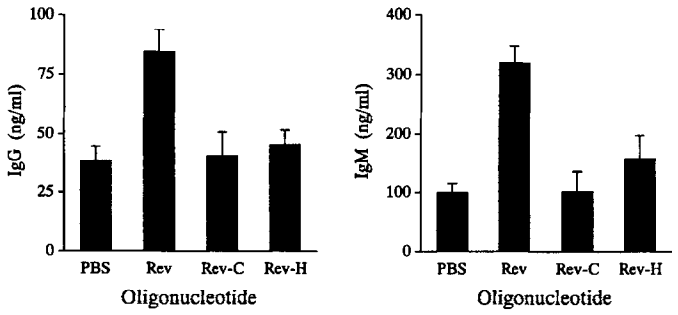

FIG. 8. IgG and IgM production measured 48 hr after injection of Rev oligonucleotide and analogs in mice. The supernatants of cell cultures were measured for IgG and IgM. The chemical modifications of the oligonucleotides are shown in Fig. 5. Bars represent the SD of triplicate cultures.

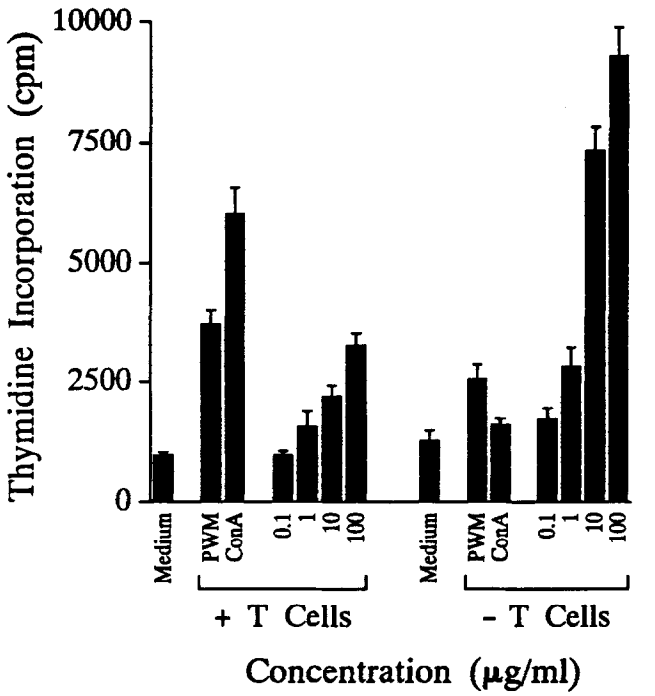

FIG. 9. Effect of T cell depletion on murine splenocyte proliferation. Splenocytes were depleted of T cells by a T-monoclonal antibody and then incubated with different concentrations of the oligonucleotide GEM 91. Results are means ± SD of triplicate cultures.

immune stimulatory effects compared with the phosphorothioate Rev oligonucleotide.

How can oligonucleotides cause stimulation of lymphocytes? DNA and structurally related synthetic oligonucleotides and polynucleotides have been shown to stimulate the lymphocytes [24, 25], but the mechanism is still not fully understood. B cells are usually activated from the resting state by antigen binding to surface immunoglobulin. In mice, activation can also be modulated by physiological mediators, such as IL-2, IL-4, γ-interferon, and non-physiological mitogens, such as LPS, ConA, and PWM. However, in our experiments, no significant induction of IL-2 was detected in *in vivo* oligonucleotide-treated mice (data not shown). T cell-depleted splenic lymphocytes did not decrease the level of proliferation. These were in agreement with other reports [14, 23]. In addition, certain oligonucleotides can even cause pronounced splenomegaly in athymic nude mice [22]. These results suggest that the immune stimulatory effect of oligonucleotides is dependent on B cells and independent of T cells. The stimulation of B cells seems to be non-specific since the activated cells did not produce anti-oligomer antibodies in response to the incubation with phosphorothioate oligonucleotides (data not shown). There are indications that natural killer cells and macrophages can be activated by oligonucleotides, especially those containing the CpG motif [15]. The reduction of the immune stimulatory effect by the modified analogs (GEM 91-C, GEM 91-H, Rev-C, and Rev-H) may not result from different biodistribution or accumulation in the spleen. Our pharmacokinetic studies have shown that phosphorothioate, chimeric, hybrid, and loop oligonucleotides have the same biodistribution in spleen and other tissues when injected intravenously ([19, 20], and unpublished results). The reduction of the immune stimulatory effect by the oligonucleotides may not result from either different levels of oligonucleotide uptake or intracellular concentration, since earlier studies demonstrated that cellular uptake of these analogs is compatible *in vitro* [26, 27]. It is possible that lymphocyte activation could arise from a more discrete protein interaction on the cell membrane or following entry of the oligonucleotide into the cell, since oligonucleotides can bind to some proteins in a non-specific manner and modification of the oligonucleotide backbone may affect their protein binding properties. The mechanism of action of oligonucleotides may be a novel mechanism, and further characterization of these events will be of interest.

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