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# Effects of Synthetic Oligonucleotides on Human Complement and Coagulation*

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**ABSTRACT.** Oligodeoxynucleotide phosphorothioates (PS-oligos) are being studied as novel therapeutic agents based on their ability to inhibit gene expression. Preclinical studies produced unanticipated complement and coagulation effects in monkeys receiving high-dose PS-oligo. In the present *in vitro* studies, PS-oligo inhibited normal human blood clotting as well as subsequent assays for prothrombin fragment $PF_{1+2}$ and hemolytic complement. PS-oligo treatment of normal donor plasma produced concentration-dependent prolongations of clotting times, with the activated partial thromboplastin time more sensitive than prothrombin time or thrombin clotting time. PS-oligo treatment of normal donor serum similarly reduced hemolytic complement activity in a concentration-dependent manner. Reduced hemolysis correlated with increased levels of complement fragment C4d. The anti-heparin drug protamine sulfate inhibited *in vitro* effects of PS-oligo in both complement and coagulation assays, suggesting that charged residues in internucleotide linkages of PS-oligo mediated the observed activities. Therefore, oligonucleotides with varying internucleotide linkages, nucleotide sequence, or secondary structure were compared. Both complement and coagulation effects appeared to be independent of nucleotide sequence but were strongly related to the nature of internucleotide linkages. Several of these modified oligonucleotides have been shown previously to retain potent antisense activity and thus may represent viable alternatives for antisense therapeutics. BIOCHEM PHARMACOL 53;8:1123–1132, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** antisense oligodeoxynucleotide; phosphorothioate; complement; coagulation

Synthetic oligonucleotides with sequence complementary to a targeted gene's mRNA transcript can suppress gene expression in a variety of experimental models [1–3]. Oligodeoxyribonucleotides synthesized with a "natural" phosphodiester backbone are unstable in biological systems due to degradation by ubiquitous nucleases [4, 5]. Synthetic oligonucleotide analogs with modified backbones have been developed in attempts to improve biological activity and pharmacokinetic profiles of antisense constructs [6–11]. PS-oligos¶ (Fig. 1) demonstrate improved stability *in vitro* and *in vivo* [5, 12–14] and are under investigation as potential therapeutic antisense agents. Several promising antisense PS-oligos are currently being tested in phase I/II trials for treatment of viral infections [15] and cancer [16].

Unanticipated, sequence-independent effects of PS-oligos have been observed during preclinical studies in monkeys. High-dose intravenous bolus administration of PS-oligos (≥5 mg/kg/10 min) to Rhesus monkeys [17] produced dose-dependent acute changes in blood pressure and heart rate, with concurrent blood samples demonstrating decreased serum hemolytic complement and increased circulating complement fragment C5a levels. In parallel, blood cell counts showed dose-related leukopenia (predominantly granulocytopenia) that rapidly resolved. These observations were considered to be consistent with intravascular complement activation [17]. Other groups have reported similar cardiovascular [18] and additional coagulation [19] effects following administration of unrelated antisense PS-oligos to monkeys. Hemodynamic changes and abnormal complement or coagulation parameters have been reported only with PS-oligo delivery protocols exceeding the dose rates proposed for current human trials, and the abnormalities reported for experimental monkeys were generally short-lived and of uncertain impact for overall hemostasis. Nonetheless, bolus infusions of high-dose PS-oligos have (albeit rarely) coincided with sudden mortality

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¶ *Abbreviations:* Bb, cleavage product of complement protein B; C4d, cleavage product of complement protein C4; $CH_{50}$, serum dilution for complement hemolysis equaling 50% of maximum lysis; $PF_{1+2}$, prothrombin fragment $PF_{1+2}$; PS-oligo, oligodeoxyribonucleotide containing phosphorothioate backbone linkages; PT, prothrombin clotting time; PTT, activated partial thromboplastin clotting time; and TCT, thrombin clotting time.

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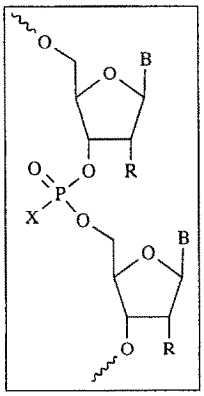
Phosphorothioate Oligo (PS-Oligo) 5'-CTCTCGCACCCATCTCTCTCCTTCT-3'

Self-stabilized Oligo 5'-CTCTCGCACCCATCTC TCT CC$^{T}$ T
3'-GAG AGA GG$_{T}$C

Hybrid Oligo 5'-**C*U*C*U***CGCACCCATCTCTCTCC**U*U*C*U***-3'

Chimeric Oligo 5'-C^T^C^T^CGCACCCATCTCTCTCC^T^T^C^T-3'



Phosphodiester Oligo: R = H; X = $O^-$

Phosphorothioate Oligo: R = H; X = $S^-$

Self-stabilized Oligo: R = H; X = $S^-$

Hybrid Oligo: R = O-$CH_3$ (*) or H; X = $S^-$

Chimeric Oligo: R = H; X = $CH_3$ (^) or $S^-$

**FIG. 1. Sequence and backbone modifications of synthetic oligonucleotide analogues. The bases shown in bold with (*) in the hybrid sequence represent 2'-O-methylribonucleoside. The symbol (^) in the chimeric sequence represents methylphosphonate internucleotide linkage.**

in treated monkeys [17, 18], the precise mechanism of which remains obscure.

In the present study, we demonstrate that the previously observed *in vivo* effects of PS-oligo on complement and coagulation in monkeys can be approximated by assays using normal human blood samples treated *in vitro* with PS-oligo. The reference PS-oligo was GEM 91®, a 25-mer antisense phosphorothioate oligodeoxyribonucleotide (Fig. 1) complementary to the initiation codon region of the HIV-1 *gag* gene, which has been shown to inhibit HIV-1 replication and is currently being tested in humans [15, 20]. Results suggest that the primary mechanism of both complement and coagulation effects depends on the unique chemical nature of the PS-oligo backbone but not on nucleotide sequence, in agreement with previous *in vivo* monkey studies [17]. *In vitro* data suggest that the potentially adverse effects of PS-oligo may be neutralized by pharmaceutical preparations of protamine sulfate, a polycationic protein. Additional data demonstrate that antisense oligonucleotides with different backbone structures (internucleotide linkages) exhibit reduced complement and coagulation effects. The results provide several practical approaches for optimizing therapeutic applications of antisense oligonucleotides.

## MATERIALS AND METHODS

### *Oligonucleotides*

GEM 91® PS-oligo and modified oligonucleotides (Fig. 1) were synthesized using an automated DNA synthesizer (Biosearch model 8800, Bedford, MA) on a 1 mM scale. Protocols for synthesis, deprotection, and purification were as reported previously for the phosphorothioate [21], chimeric [22], hybrid [10], and self-stabilized [9] oligonucleotides.

For *in vitro* blood fraction assays, lyophilized oligonucleotides were dissolved in sterile normal saline [0.9% (w/v) NaCl] to make stock concentrations of between 2 and 10 mg/mL and were stored at 4°. These stock solutions comprised <10% (v/v) of blood, plasma, or serum samples analyzed in the reported experiments, with parallel controls including blood fractions treated with identical volumes of saline alone.

### *Other Reagents*

Protamine sulfate (Elkins-Sinn, Inc. Cherry Hill, NJ) was used as a 10 mg/mL USP injectable solution. Porcine heparin (Sigma, St. Louis, MO; sodium salt) was dissolved in sterile normal saline for a concentration of 10 mg/mL (1430 USP K-1 units/mL). Mouse genomic DNA was prepared from young adult (6- to 12-week-old) BALB/c splenocytes using standard protocols [23].

### *Normal Human Blood, Serum, and Plasma*

Venous blood was collected from healthy adult volunteers employed at the University of Alabama at Birmingham. No individual was known to have donated more than 100 cc whole blood over the preceding 30-day period or to have undergone pheresis in the preceding 3-month period. All donors gave verbal histories negative for both prescription

and non-prescription medications for the previous 48-hr period.

Serum for hemolytic complement assays was prepared by collecting blood into siliconized vacutainers without commercial additives (Becton Dickinson No. 6430). Blood was allowed to clot at room temperature for 30 min, was chilled on ice for 15 min, and then was centrifuged at 4° to separate serum. Harvested serum was kept on ice until assay.

Plasma for clotting time assays was prepared by collecting blood into siliconized vacutainers with 3.8% sodium citrate (Becton Dickinson No. 367705), followed by two centrifugations at 4° to prepare platelet-poor plasma. Plasma aliquots were kept on ice, spiked with various test compounds, and either tested immediately or frozen quickly on dry ice for subsequent storage at −20° for up to 4 days prior to coagulation assays.

In some experiments, siliconized vacutainers (Becton Dickinson No. 6430) without commercial additives were spiked with small aliquots (< 0.25 mL/tube) of test compounds prior to venipuncture, with final test compound concentrations in the collected whole blood calculated by the estimated vacutainer final draw volume.

### *Coagulation Assays*

$PF_{1+2}$, released when prothrombin is cleaved to thrombin during coagulation, was quantitated using a commercial sandwich ELISA (Baxter Dade, Miami, FL) according to the manufacturer's recommended protocol. Normal levels of $PF_{1+2}$ in citrated plasma are 2.2 nM. Levels in serum prepared from normal donor blood samples allowed to clot *ex vivo* are reported here as > 400 nM, which is the upper limit of quantitation for the sample dilutions that were tested in the reported $PF_{1+2}$ assays.

The PTT assay was performed with citrated normal donor plasma in duplicate on an Electra 1000C (Medical Laboratory Automation, Mount Vernon, NY) according to recommended procedures using Actin FSL (Baxter Dade) and 25 mM $CaCl_2$ to initiate clot formation, which was measured photometrically. Normal plasma PTT values ranged from 27 to 39 sec. PT was measured in a similar manner using the Innovin reagent (Baxter Dade) to initiate clotting of citrated plasma. Normal plasma PT values ranged from 10.0 to 12.6 sec. TCT was determined similarly following addition of the Thrombin-Prest reagent (American Bioproducts, Parsippany, NY) to citrated plasma. Normal plasma TCT values ranged between 12 and 16 sec.

### *Complement Assays*

A standard $CH_{50}$ assay for complement lysis of sheep red blood cells (Colorado Serum Co., Denver, CO) sensitized with anti-sheep red cell antibody (hemolysin, Diamedix, Miami, FL) was performed as described [24], using duplicate determinations of at least five dilutions of each test serum sample. Except where specifically noted, vehicle control or test compounds were mixed initially with undiluted serum and the samples subsequently diluted 1:50 in standard $CH_{50}$ assay buffer for use in the hemolytic assay. Maximal hemoglobin release was determined following hypotonic lysis in sterile deionized water, and spontaneous release by incubation of red cells in assay buffer without serum; spontaneous release was less than 2% of maximum in all reported experiments. Hemoglobin release into cell-free supernatants was quantitated spectrophotometrically at 541 nm and $CH_{50}$ units/mL extrapolated [24]. Normal donor serum activity ranged between 290 and 360 units/mL. Estimates of alternative complement pathway activity were obtained in a modified $CH_{50}$ assay substituting non-sensitized rabbit red blood cells (Colorado Serum Co.) as targets for lysis and calculating units/per milliliter for 20% total lysis; normal donor serum activity ranged between 95 and 130 units/mL. In some experiments, single serum dilutions were tested in triplicate, with results presented as the mean percent of total red cell lysis.

Commercial ELISA kits (Quidel, San Diego, CA) were used to quantify complement fragments C4d (classical pathway specific) and Bb (alternative pathway specific) in plasma or serum samples, using the manufacturer's recommended protocols for sample dilution and assay performance.

### *Protein Binding to Oligonucleotides*

PS-oligo was labeled with $^{32}P$ at the 5′-end using [$\gamma$-$^{32}P$]ATP (Amersham, Arlington Heights, IL) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Approximately 1 nmol of labeled PS-oligo was incubated with different concentrations of protamine in 10 mM $Na_2HPO_4$, 100 mM NaCl buffer, pH 7.5, at 37° for 1 hr. Then the reaction mixture was loaded with glycerol dye on an 8% non-denaturing polyacrylamide gel. Electrophoresis was carried out at room temperature using 50 mM Tris, 50 mM glycine buffer, pH 7.4. After electrophoresis, the gel was dried and autoradiographed using Kodak X-Omat AR film at −70°.

## RESULTS

### *Effects of PS-oligo Treatment of Human Donor Whole Blood*

The following *in vitro* experiments were designed to mimic *in vivo* treatment with PS-oligo: Normal donor venous blood was collected directly into siliconized vacutainers containing small volumes (~4% of final draw volume) of 10 mg/mL PS-oligo solution, for a final estimated *ex vivo* blood concentration of 400 μg/mL PS-oligo. Blood samples collected in this manner consistently failed to form visible clots after ≥30 min of incubation at room temperature, whereas blood collected concomitantly from the same donors into control vacutainers containing the saline vehicle alone readily formed clots. Cell-free supernatants (plasma/serum) prepared by centrifugation of the PS-oligo blood samples demonstrated low levels of $PF_{1+2}$ (Table 1), reflect-

ing the lack of significant clotting activity. The same PS-oligo supernatants demonstrated reduced hemolytic complement activity that could not be explained by general anti-coagulation based on parallel assay of supernatants from heparin-anticoagulated blood (Table 1). These *in vitro* studies support the general effects of PS-oligo on complement and coagulation previously observed following intravenous dosing of monkeys [17].

### *Effect of PS-oligo on Complement Activity Following* In Vitro *Serum Treatment*

Normal rhesus monkey serum and normal human donor serum were treated *in vitro* with high-concentration (500 μg/mL) PS-oligo and subsequently analyzed for both classical and alternative pathway complement hemolytic activity (Table 2). Because "natural" DNA has been reported to specifically bind complement component C1q and activate the classical complement pathway [25, 26], the PS-oligo was studied in parallel with equivalent concentrations of mouse genomic DNA. The data in Table 2 show that genomic "natural" DNA had minimal measurable effects on complement hemolytic activity, whereas PS-oligo resulted in reduced serum hemolytic activity against either classical or alternative complement pathway targets. The apparent disparity between these results and those previously reported by other workers for phosphodiester DNA is probably due to differences in experimental design; whereas Jiang and co-workers [26] reported DNA-mediated complement activation in dilute serum samples [e.g. 10% serum, 90% buffer (v/v)], all of our studies have been conducted with minimally diluted sera [e.g. 90% serum, 10% test solution (v/v)]. Studies directly comparing complement effects of PS-oligo with a synthetic phosphodiester oligodeoxynucleotide of the same sequence and length (see Fig. 6) support this conclusion.

Prior incubation of PS-oligo with serum as in Table 2 was not required for efficient inhibition of hemolysis, and roughly equivalent reductions of hemolysis were observed when PS-oligo at 10-fold lower concentrations was added only to the $CH_{50}$ assay buffer. These results should be considered in the context that serum hemolytic complement is inactivated rapidly at 37°, and the serum pretreatment incubation (10 min) was shorter than that for subsequent measurement of red cell hemolysis (30 min). One interpretation of these results is that the binding affinity between complement components and PS-oligo was similar to that between complement components and the "physiologic" activating targets used as indicators in $CH_{50}$ assays (e.g. opsonized red cells). A second interpretation is that PS-oligo directly interfered with formation of the terminal complement membrane attack complex in target red cell membranes.

PS-oligo pretreatment of normal human serum *in vitro* demonstrated a concentration-dependent reduction in hemolytic complement activity (Fig. 2A) with parallel increases in serum concentrations of complement fragment C4d (Fig. 2B) but with relatively minor changes in complement fragment Bb (Fig. 2C). The present *in vitro* assays' normal means (3 SD range) of concentrations for these two fragments in serum were 4.6 (0 to 9.7) μg/mL for C4d and 4.1 (0 to 8.8) μg/mL for Bb. These results suggest that PS-oligo activates serum complement, primarily via the classical (C4d-specific) pathway, thereby consuming complement components necessary for subsequent expression of full serum hemolytic activity against either classical or alternative pathway-specific targets. Because C4d generation occurs earlier in classical pathway activation than C5a, and because C5a is generated by both classical and alternative pathway activation, these data corroborate and extend previous observations of serum C5a increases in PS-oligo-treated monkeys [17].

### *Effect of PS-oligo on Coagulation Activity by Plasma Treatment* In Vitro

PS-oligo added to platelet-depleted, normal donor citrated plasma *in vitro* prolonged measured plasma clotting times in

**TABLE 1. Effect of PS-oligo in whole blood**

| Additives (400 μg/mL) | | | $PF_{1+2}$ (nM) | Hemolytic complement (% of control) | |
|---|---|---|---|---|---|
| PS-oligo | Heparin | Protamine | | Classical | Alternative |
| – | – | – | >400 | (100) | (100) |
| + | – | – | 11.9 | 44 | 24 |
| – | + | – | 13.7 | 110 | 90 |
| – | – | + | >400 | 100 | 85 |
| – | + | + | >400 | 97 | 73 |
| + | – | + | >400 | 103 | 99 |

Blood was collected into vacutainers previously spiked with additives for the indicated estimated final blood concentrations, with normal saline vehicle alone as the control (–) additive. After incubation at room temperature for 30 min to allow clotting, blood samples were centrifuged, and the resultant supernatants were assayed by ELISA for $PF_{1+2}$ and by hemolytic assays for classical and alternative complement pathway activity. Complement results are presented as percent of control (saline alone) hemolytic units/mL.

**TABLE 2. Hemolytic complement activity after *in vitro* treatment of human and monkey serum**

| | Hemolytic activity (units/mL) | | | |
|---|---|---|---|---|
| | Human serum | | Rhesus monkey serum | |
| | Classical | Alternative | Classical | Alternative |
| Control | 352 (1.00) | 118 (1.00) | 173 (1.00) | 130 (1.00) |
| PS-oligo | 177 (0.50) | 51 (0.43) | 130 (0.75) | 73 (0.56) |
| dsDNA | 314 (0.89) | 109 (0.92) | 175 (1.01) | 127 (0.98) |

Human normal donor and normal rhesus monkey serum were treated at 37° for 10 min with either no addition (control), 500 μg/mL of PS-oligo, or 500 μg/mL of mouse genomic DNA (dsDNA). Sera were then diluted 1:50 and used in standard hemolytic assays for classical ($CH_{50}$) or alternative complement activity. Numbers in parentheses show the fraction of control hemolytic units/mL.

a concentration-dependent manner (Fig. 3). The PTT (Fig. 3B) was the most sensitive assay, with 50% prolongation of control obtained at PS-oligo concentrations of approximately 4 μM (33 μg/mL), compared with 24 μM (210 μg/mL) and 48 μM (420 μg/mL) for the corresponding TCT (Fig. 3C) and PT (Fig. 3A), respectively, in the experiments shown. This pattern of clotting time prolongation is consistent with interference of contact activation by PS-oligo. The prolonged PTT of PS-oligo-treated normal plasma was not reversed by addition of an equal volume of untreated normal plasma (data not shown), indicating that PS-oligo behaved as a fast-acting fluid-phase anticoagulant.

### *Reversal of PS-oligo Effects by Protamine*

The observed anti-coagulant effects of PS-oligos resemble those of heparin, a complex proteoglycan containing charged sulfur residues. The heparin-neutralizing drug protamine sulfate reversed both anti-coagulant and anti-complement effects of PS-oligos in whole blood (Table 1). Protamine further neutralized PS-oligo effects on complement in normal serum (Fig. 2) and on coagulation in normal citrated plasma (Fig. 4) when protamine was added to blood samples prior to PS-oligo addition. Results of multiple experiments using blood samples from different normal donors indicated that the 25-mer PS-oligo was optimally neutralized at a ratio of 0.5 mg protamine/1 mg PS-oligo, provided that the protamine concentration in plasma did not exceed 100 μg/mL, a concentration at which protamine alone prolonged the PTT (Fig. 4). At lower ratios (e.g. 0.25 mg protamine/1 mg PS-oligo, Fig. 2), protamine was unable to effectively neutralize the activity of PS-oligo.

To test the hypothesis that protamine formed complexes with PS-oligo that could neutralize PS-oligo activity in complement and coagulation assays, gel mobility shift experiments were performed using radiolabeled PS-oligo mixed with various amounts of protamine sulfate (Fig. 5A). The results indicate that protamine interacts with PS-oligo in a concentration-dependent manner, with saturation binding of protamine to PS-oligo estimated at a 0.9 molar

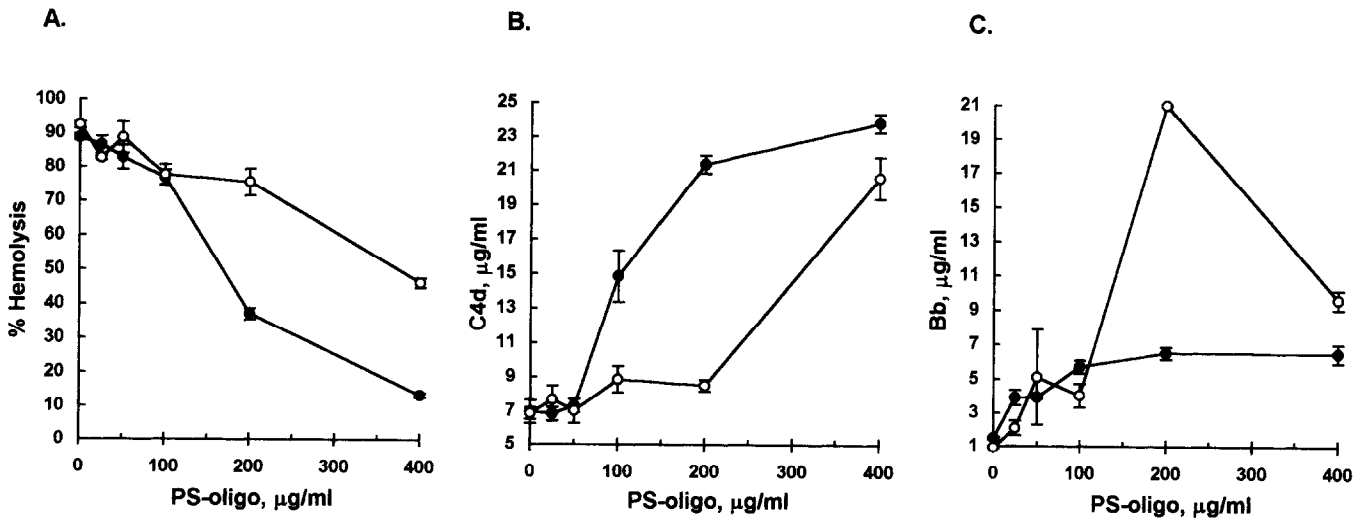



FIG. 2. **Effect of PS-oligo on serum complement activity. Normal donor serum was mixed either with saline alone (●) or with 100 μg/mL protamine (○), and then spiked with the indicated concentrations of PS-oligo, incubated at 37° for 15 min, and placed on ice prior to assay. (A) Hemolytic complement by modified $CH_{50}$ assay using a single dilution (1/250) of each sample. (B) ELISA quantitation of serum complement fragment C4d. (C) ELISA quantitation of serum complement fragment Bb. Results for all three assays are means ± SD of triplicates.**

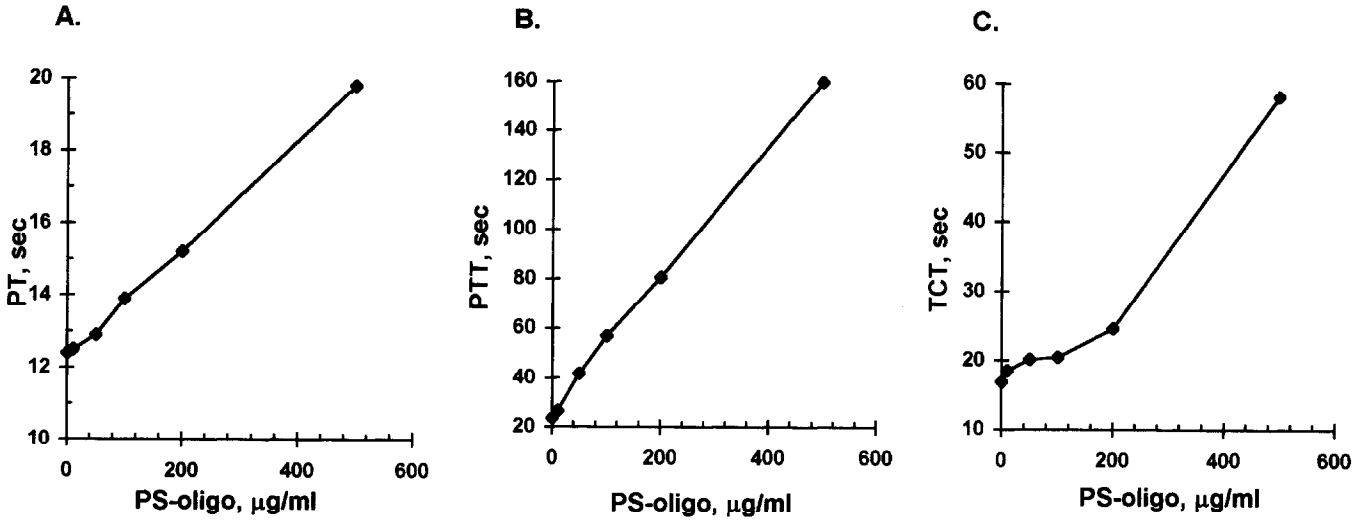



**FIG. 3. Effect of PS-oligo on plasma clotting times. Normal donor citrated plasma was spiked with the indicated concentrations of PS-oligo at 4° prior to assay of clotting times: (A) PT, (B), PTT, and (C) TCT. Data points are means of duplicate determinations.**

ratio (based on an average protamine mol. wt = 4500); this ratio approximates the optimal neutralization ratio estimated by the above coagulation and complement functional assays. Under the same conditions, a control phosphodiester oligonucleotide (of sequence complementary to the PS-oligo) demonstrated no detectable association with protamine, suggesting that the interaction depends on sulfur substitution in the PS-oligo backbone.

Interactions between protamine and PS-oligo were of a non-specific charge neutralization type that were reversed readily by the addition of competing polyanions such as sodium dodecyl sulfate and heparin (data not shown). The estimated $K_d$ from gel mobility shift experiments for the binding of protamine to PS-oligo was 6 μM (Fig. 5C). Competitive titration of protamine–PS-oligo complexes with either DNA or RNA oligonucleotides with sequence complementary to the PS-oligo resulted in dissociation of PS-oligo from protamine and concomitant formation of oligonucleotide duplexes (Fig. 5B). These results suggest that binding of PS-oligo to at least some positively charged proteins may not interfere with nucleotide sequence-specific hybridization of PS-oligo with its target mRNA.

### *Effects of Oligonucleotide Modifications on Complement and Coagulation*

The above protamine results were consistent with the hypothesis that the activity of PS-oligo in complement and coagulation assays was at least partially dependent upon charged sulfur residues of the PS-oligo backbone. To test this hypothesis, the effects on complement and coagulation of various oligonucleotides differing from the reference PS-

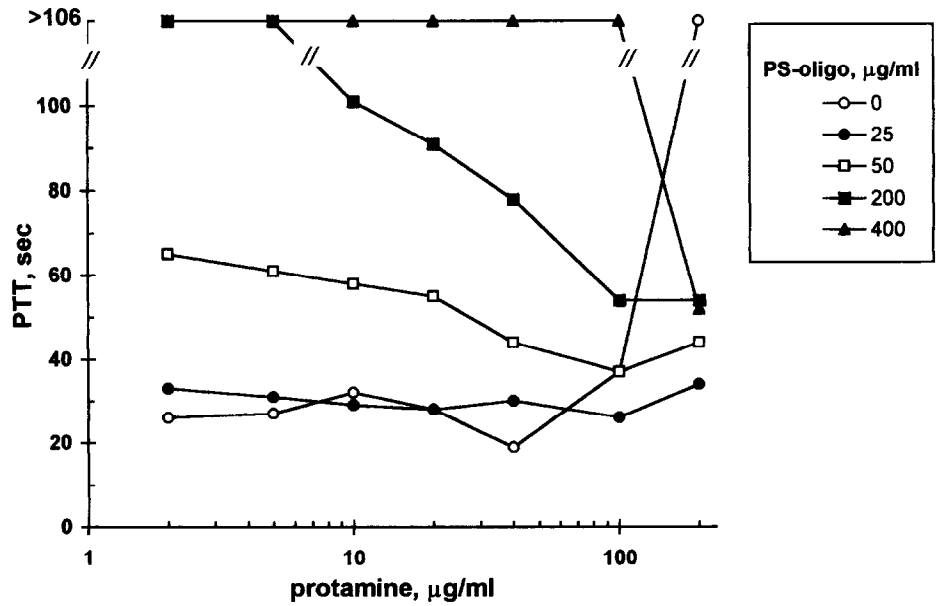



**FIG. 4. Effect of protamine on clotting prolongation by PS-oligo. Citrated normal donor plasma was spiked with various concentrations of PS-oligo and frozen until assay of PTT, with or without prior addition of the indicated final concentrations of protamine (x-axis). Clot formation was monitored for a maximum of 106 sec, after which data were recorded as "no clot detected."**

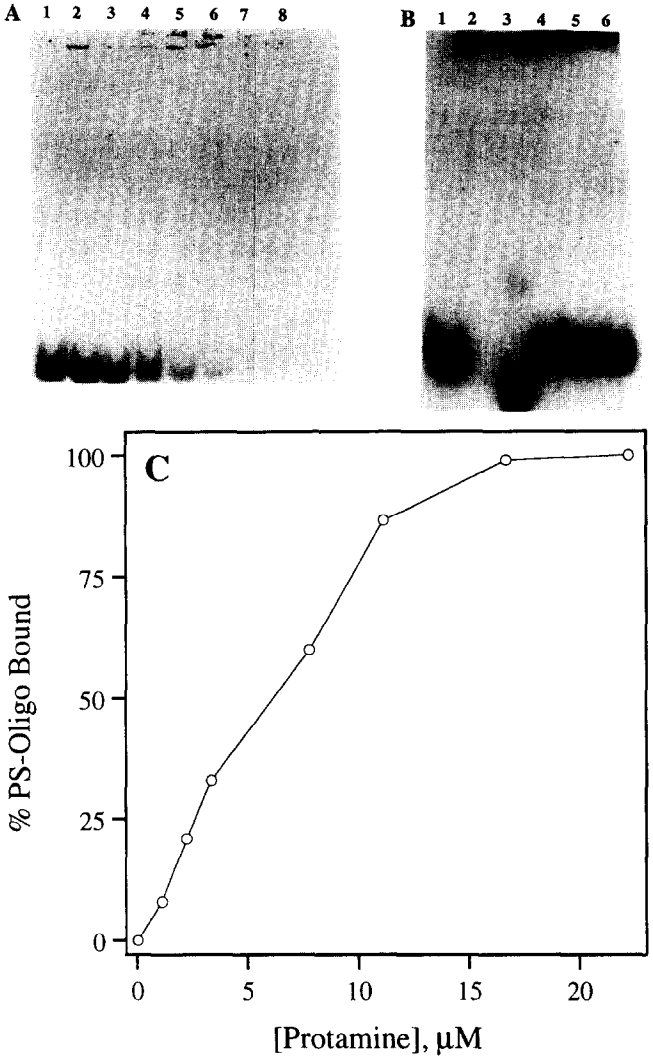



**FIG. 5. (A) Autoradiogram showing binding of PS-oligo to protamine. Lane 1 contains PS-oligo alone and lanes 2–8 contain PS-oligo in the presence of 5, 10, 15, 35, 50, 75, and 100 mg of protamine, respectively. The decrease in intensity of the PS-oligo band with increasing concentration of protamine is due to binding of PS-oligo to protamine. Note that protamine does not enter the gel due to its high positive charge. (B) Autoradiogram showing PS-oligo binding to its complementary DNA strand in the presence of protamine. Lane 1, PS-oligo alone; lane 2, PS-oligo plus protamine; lane 3, complementary (to PS-oligo) DNA oligonucleotide (phosphodiester backbone) plus protamine; lanes 4–6, PS-oligo plus protamine and complementary DNA oligonucleotide at ratios of 1:1, 1:2, and 1:4 (PS-oligo to complementary DNA oligonucleotide), respectively. (C) Graph showing the binding of PS-oligo to protamine as a function of increasing concentration of protamine, from which the $K_d$ was determined. Data were obtained by densitometric analysis of Fig. 5A.**

oligo in either base sequences or the nature of internucleotide linkages (Fig. 1) were tested. Figure 6 shows that a random sequence PS-oligo (scrambled) of the same size as the reference 25-mer PS-oligo produced similar reduction of hemolytic complement, whereas a phosphodiester oligonucleotide with the same sequence as the reference PS-

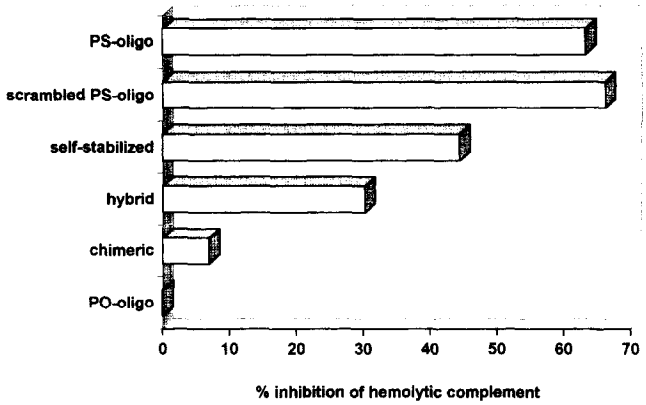



**FIG. 6. Effects of modified oligonucleotides on hemolytic complement. Normal donor serum was incubated for 10 min at 37° with 200 μg/mL of the indicated oligonucleotides prior to assay of hemolytic complement. Data are presented as percent inhibition by reference to control serum treated with saline vehicle alone.**

oligo had little or no effect. These results support previous results obtained using genomic DNA (Table 2). The self-stabilized oligonucleotide (33-mer) containing a 3′ loop structure, the hybrid oligonucleotide phosphorothioate containing terminal 2′-O-methyl-RNA residues, and the chimeric oligonucleotide containing terminal non-ionic methylphosphonate linkages (see Fig. 1 for structures) were progressively less active than PS-oligo in reducing hemolytic complement activity (Fig. 6). The effects of these modified oligonucleotides were concentration dependent, and this relative order of activity has been observed consistently in independent experiments using either whole blood or serum treatments *in vitro*. The chimeric oligonucleotide at concentrations up to 500 μg/mL has never been observed to produce greater than 40% reduction of hemolytic complement in over six independent experiments with different normal donor sera.

A similar activity hierarchy for the modified oligonucleotides was observed in coagulation assays performed with *in vitro* treated normal human plasma. Table 3 presents the estimated oligonucleotide concentrations producing 50%

**TABLE 3. Concentrations of modified oligonucleotides prolonging clotting times by 50% *in vitro***

| | Oligonucleotide concentration (μg/mL) producing 50% prolongation | | |
|---|---|---|---|
| | PTT | TCT | PT |
| PS-oligo | 18 | 130 | 330 |
| Self-stabilized | 18 | 145 | >500 |
| Hybrid | 35 | 235 | >500 |
| Chimeric | 43 | 265 | ≫500 |

Aliquots of normal donor citrated plasma were spiked with concentrations of each oligonucleotide analogue from 10 to 500 μg/mL and then assayed in standard clinical clotting assays for PTT, TCT, and PT. Means of duplicate measurements at five concentrations were used to extrapolate concentrations producing 50% prolongation in each clotting time assay. Absolute values differ from those in Fig. 3 due to plasma donor variations.

prolongation of clinical clotting times when added to normal donor citrated plasma. As previously observed (Fig. 3), PTT assays were more sensitive to prolongation by all oligonucleotide analogs than either TCT or PT assays. The chimeric oligonucleotide demonstrated the least effect on coagulation, requiring a 2-fold or higher concentration than the reference PS-oligo to produce an equivalent prolongation of PTT.

## DISCUSSION

In the past decade, there has been an explosive growth in the number of well-defined genes that are directly implicated in the pathogenesis of human diseases, both acquired and inherited. For many of these diseases, it seems reasonable to propose that selective inhibition of the identified pathogenic gene product will yield improved treatment options, and in some cases potential cures, with the prediction of minimal treatment-associated toxicities. Antisense oligonucleotides are reported to exhibit the desired gene-specific "knock-out" effects in a number of *in vitro* and *in vivo* experimental models [27, 28]. Antisense oligonucleotides offer a theoretical advantage over other approaches to rational drug design, because antisense design does not require detailed knowledge of the function–structure properties of the protein, relying instead upon nucleotide sequence.

However, "natural" phosphodiester oligonucleotides are generally unsuitable for use as therapeutic agents due to rapid degradation by ubiquitous exo- and endonucleases. Hence, PS-oligos have been developed in which sulfur is substituted for a non-bridging oxygen of the "natural" internucleotide linkage. PS-oligos have been the analogs of choice for many preclinical studies and clinical trials [14–16, 29] because they exhibit substantial nuclease resistance but are also efficiently taken up by cells [5, 12, 13].

High-dose bolus infusions of PS-oligo in non-human primates produced multiple unanticipated effects as measured by standard clinical laboratory assays [17–19]. In addition to the previously reported effects on circulating complement, administration of 5–20 mg/kg doses of PS-oligo by 10 min i.v. infusion [17] was associated regularly with appreciable (greater than 50%) prolongation of baseline PTT, in a dose-related manner. PTs were prolonged to a lesser extent, clottable fibrinogen was variably decreased, fibrin degradation products were increased markedly, and plasma D-dimers were increased slightly (Agrawal *et al.*, unpublished data). These results suggest, but do not prove, that the intrinsic pathway of blood coagulation was affected. Such changes were rapidly reversible, and the monkeys exhibited no overt bleeding episodes or other adverse effects directly attributable to inhibition of coagulation.

The data presented here show that PS-oligo can similarly affect complement and coagulation when added to whole blood, plasma, or serum *ex vivo*. Demonstration of these effects in cell-free serum and plasma indicate that they are unrelated to antisense activity. Such antisense-independent effects were also suggested by the previous *in vivo* study in which a control non-antisense PS-oligo was administered to monkeys [17].

The current study further supports the hypothesis that complement and coagulation effects observed with PS-oligos are primarily attributable to the chemical nature of the phosphorothioate internucleotide linkage. First, PS-oligos with random nucleotide sequences produced similar effects *in vivo* [17] and *in vitro*. Second, oligonucleotides with similar antisense nucleotide sequences but with varying backbone chemistries (self-stabilized, hybrid, and chimeric oligonucleotides) demonstrated differential effects on both complement and coagulation activities that cannot be explained by either their individual nuclease resistance or antisense potency relative to the PS-oligo [9, 10, 22].

Neutralization by protamine of the PS-oligo effects on both complement and coagulation provides indirect support that phosphorothioate backbone is pivotal for both activities. In the PS-oligo, a sulfur is substituted for an oxygen, changing the charge distribution on the phosphorothioate linkage as compared to the phosphodiester linkage. The major resonance form of the phosphorothioate is that in which sulfur carries a net negative charge [30]. Localization of this negative charge on the sulfur residue probably explains the relatively high binding affinity of PS-oligo to polycationic protamine relative to phosphodiester oligonucleotide under the same experimental conditions. We suggest that the association between protamine and PS-oligo effectively neutralizes the negatively charged PS-oligo residues that are responsible for most of the observed complement and coagulation activities, similar to what is believed to occur between heparin and protamine.

Jiang and coworkers [26, 31] have published studies of interactions between DNA and the complement component C1q which activate the classical complement cascade. DNA was reported to bind to specific cationic peptides derived from collagen-like domains of C1q and, conversely, these same C1q peptides could inhibit binding of DNA to intact native C1q; the same C1q peptides were also shown to bind heparin. It is conceivable that the PS-oligo effects on complement reported in this study involve similar or identical mechanisms. The data are consistent with complement activation by PS-oligo at the level of either C1q, C2, or C4. Results presented here further indicate that phosphorothioate (PS) residues may potentiate interactions between oligodeoxynucleotides and complement, because we observed minimal effects on complement activity by phosphodiester analogues assayed in parallel with PS-oligo. The greater sensitivity of phosphodiester DNA to nuclease degradation as compared to PS-oligo may explain our results that were obtained after adding oligonucleotides to previously undiluted sera. An alternative mechanism is that PS-oligos have a significantly higher binding affinity for complement proteins than phosphodiester nucleotides which translates into greater measured complement effects,

analogous to the reported phosphorothioate magnification of B lymphocyte mitogenesis for unmethylated CpG oligonucleotide motifs [32]. Additional studies will be required to resolve such questions of nuclease resistance versus oligonucleotide charge.

Previous reports of coagulation modulation by phosphodiester oligonucleotides are, to our knowledge, limited to the special case of sequence-specific "thrombin aptamers" [33, 34]. The most potent thrombin aptamer reported is the 15-mer phosphodiester oligonucleotide GGTTGGTGTGGTTGG [33], which binds thrombin but not other serine proteases in a sequence-specific manner, and which efficiently inhibited clotting reactions both *in vitro* and *in vivo*. In contrast, the interaction of PS-oligos with proteins, in general, is not sequence specific but related to the chemical nature of the phosphorothioate backbone [35]. Recent studies demonstrate that binding of the PS-oligos to serum and plasma proteins depends on the chemical nature of the backbone and its concentration [5, 36]. Binding affinity of PS-oligo to major serum proteins is in the order fibrinogen > γ-globulins > albumin (Kandimalla ER and Agrawal S, unpublished data). These results suggest that the observed hemodynamic effects are more related to non-sequence-specific association of PS-oligos of certain chain length with proteins [17].

Finally, the data presented here suggest that the therapeutic use of PS-oligo antisense reagents may be associated with increased risk of acute complications caused by systemic complement activation and/or blood anticoagulation. *In vivo* complications reported to date have been observed only in primates and only following bolus intravenous doses exceeding 5 mg/kg [17, 18]. These effects are related to the plasma concentration of PS-oligo and can be circumvented by administering the required concentration of PS-oligo by slow i.v. infusion, as currently employed in ongoing clinical trials, or by formulations engineered to minimize such activities.

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