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Influence of Backbone Chemistry on Immune Activation by Synthetic Oligonucleotides

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ABSTRACT. Depending on base sequence, DNA displays immunological activities relevant to the design of novel therapeutic agents. To determine the influence of backbone structure on these activities, we tested a series of synthetic phosphodiester and phosphorothioate oligonucleotides in *in vitro* cultures of murine spleen cells. These compounds were 30 bases long and consisted of either a single base or an immunostimulatory sequence (AACGTT) flanked on 5′ and 3′ ends by 12 nucleotides of each base. Cell activation was assessed by both thymidine incorporation and expression of cell surface CD69; production of interleukin-6 and interleukin-12 was used as a measure of cytokine stimulation. In these assays, phosphorothioate oligonucleotides induced much higher levels of proliferation, CD69 expression, and cytokine production than the comparable phosphodiester compounds and had activity at lower concentrations. The sequence for optimal stimulation by phosphorothioates varied among responses, however. For example, whereas compounds containing an immunostimulatory sequence all induced similar levels of proliferation and CD69 expression, cytokine production was greatest with compounds with dA and dT flanks. Furthermore, while single base dG oligonucleotides stimulated proliferation as both phosphodiesters and phosphorothioates, they failed to stimulate cytokine production. Together, these findings indicate that base sequence as well as backbone chemistry influence immune activation by synthetic oligonucleotides, with the effects varying among responses. While suggesting differences in the structure–function relationships of nucleic acids in their immune activities, these findings also raise the possibility of the design of agents with specific patterns of immune modulation. BIOCHEM PHARMACOL **58**;12:1981–1988, 1999.

KEY WORDS. DNA; oligonucleotides; phosphorothioate; mitogenesis; cytokine production; immune stimulation

DNA is a complex macromolecule whose immunological properties depend on base sequence [1, 2]. Although mammalian DNA is immunologically inert, DNA from bacteria has potent immune activities and can induce B cell activation as well as the production of cytokines including IL-12†, TNF-α, IL-6, IFN-α/β and IFN-γ. These activities result from short sequences, called CpG motifs or ISS, that have the general structure of two 5′ purines, an unmethylated CpG dinucleotide, and two 3′ pyrimidines [3–7]. These sequences occur much more commonly in bacterial DNA than in mammalian DNA and may function as "danger signals" to activate innate immunity [8, 9].

While suggesting a role of foreign DNA in host defense, studies on ISS also have generated interest in the use of DNA as an immunomodulatory agent. As shown in animal experiments, DNA has adjuvant effects and can enhance vaccine responses for foreign antigens as well as tumors [10–13]. Furthermore, ISS sequences in DNA vaccines may contribute to the effectiveness of these novel agents [14, 15]. In the development of DNA as an immunomodulator, initial work has focused on synthetic oligonucleotides. These compounds, consisting of an ISS in the context of flanks of varying sequences, have been synthesized as either Po or Ps molecules. Ps oligos are DNA derivatives in which a sulfur atom is substituted for one of the non-bridging oxygens in the Po backbone. This substitution alters important physical–chemical properties such as melting temperature, sensitivity to nuclease digestion, and cell entry [16–18].

Although both Ps and Po oligos have immunostimulatory properties, these compounds may differ in structure–function relationships as well as in the range of their activities. For example, whereas bacterial DNA and Po oligos fail to stimulate human B cells, Ps oligos can potently activate these cells for proliferation and antibody production. Furthermore, stimulation of murine B cell proliferation by Ps oligos is not related directly to the content of CpG motifs, suggesting that the pattern of immunostimulation by DNA may vary with backbone chemistry [19–22]. Delineating differences between Ps and Po oligos is impor-

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† *Abbreviations*: IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; CpG, cytosine guanosine dinucleotide; ISS, immunostimulatory sequence(s); Po, phosphodiester; Ps, phosphorothioate; 30mers, oligonucleotides containing 30 bases; AP, active palindrome; and oligos, oligonucleotides.

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tant not only for the design of therapeutic DNA agents but also for understanding the mechanisms by which bacterial DNA stimulates immunity.

To elucidate more completely the immune properties of DNA differing in backbone chemistry, we have compared cell activation and cytokine production induced by a series of Ps and Po oligos of the same sequence. These compounds were all 30 bases long and consisted of either a single base or an ISS in the context of 12-base runs of a single nucleotide at the 5′ and 3′ ends. In *in vitro* cultures of murine spleen cells, these compounds were tested for their ability to induce mitogenesis and cell surface CD69 expression, as well as the production of two cytokines, IL-6 and IL-12. Results of these studies indicate that backbone chemistry has an important impact on immune stimulation and that Ps and Po compounds may differ significantly in their structure–function relationships.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were purchased from the Midland Certified Reagent Co. The compounds were synthesized using cyanoethyl phosphoramidite chemistry and purified by gel filtration. The dried compounds were dissolved to a concentration of 100 μM in distilled water and were sterilized by filtering through a 0.22-μm Millex-GV filter (Millipore). O.D. 260 readings before and after filtration showed no loss of material.

Mouse Splenocyte Culture

BALB/cByJ and C3H/HeJ mice were purchased from the Jackson Laboratory and housed under conventional conditions in the animal facility of the Durham VA Hospital. Mice were killed by cervical dislocation, and their spleens were removed aseptically. Non-stromal cells were expressed with flame-sterilized, frosted-end microscope slides into serum-free RPMI 1640 medium (Life Technologies). Suspended cells were transferred to a 15-mL conical centrifuge tube, and large debris was allowed to settle. The overlying cell suspension was removed carefully and centrifuged at 400 *g* for 5 min. The cell pellet was resuspended in hypotonic lysis medium to eliminate red blood cells (1 vol. of 0.17 M Tris, pH 7.65:9 vol. of 0.16 M NH_4Cl) and repelleted. Then cells were put through two cycles of washing/centrifugation with RPMI to eliminate hypotonic medium. The final pellets were suspended in complete medium consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum (HyClone), 2 mM L-glutamine, 1 mM sodium pyruvate, and 5×10^{-5} M 2-mercaptoethanol (Life Technologies). Cells were counted using a hemacytometer, and cell concentrations were adjusted with complete medium. Cells were plated in 96-well cell culture clusters (Costar Inc.). For proliferation assays, cells were plated at $2–5 \times 10^5$ cells/well, whereas for cytokine assays, cells were plated at $1–2 \times 10^6$ cells/well.

Proliferation Assays by Tritiated Thymidine Incorporation

After 48 hr of cell culture, 0.5 μCi of tritiated thymidine (6.7 Ci/mmol, DuPont NEN) was added to each well in an additional 25 μL of serum-free RPMI medium. After incubation for 6 hr, cells were harvested on glass fiber filter paper using a Bellco Microharvester (Bellco Glass Inc.). Radioactivity was measured by scintillation spectrometry using a Packard Tri-Carb liquid scintillation system (Packard Instrument Co.). All wells were set up in triplicate, and results were expressed as mean counts/min/well.

Assay of CD69 Expression

After 20 hr of cell culture, cell surface expression of CD69 was evaluated using FITC-labeled anti-mouse CD69 monoclonal antibody (Clone H1.2F3, PharMingen) on a FACScan Flow Cytometer (Becton Dickinson). At least 10,000 cells were evaluated from each sample. LYSYS II software was used to analyze the results.

Cytokine Analysis

IL-6 and IL-12 secretion was measured by ELISA, using capture immunoassays. Culture supernatants were removed at 24 hr (IL-12) or 48 hr (IL-6) and frozen at −20° until analysis. ELISA plates (Immulon II HB, Dynex Technologies) were prepared by coating each well with 100 μL of capture antibody diluted to 5 μg/mL in PBS, pH 8.5, at 4°. For IL-6, Clone MP5–20F3 (PharMingen) was used as the capture antibody, and Clone C15.6 anti-p40 was used for the IL-12 assay (PharMingen). After overnight incubation, plates were washed with PBS, pH 7.4, using an automated plate washer (Skatron Instruments Inc.). One hundred microliters of culture supernatants diluted 1:4 in PBS, pH 7.4, containing 0.4% Tween-20 (Sigma) and 0.5% bovine serum albumin (Sigma) was added to each well. Recombinant cytokine standards (IL-6 from PharMingen, IL-12 from Genzyme) were diluted 1:2 in concentrations from 50 to 0.05 ng/mL for IL-6 and 20 to 0.02 ng/mL for IL-12 p40/70. Diluted standards in duplicate were run on each plate.

Diluted supernatants and standards were allowed to incubate for 2 hr at room temperature. After washing of the plates, 100 μL of diluted biotinylated detection antibody was added to each well. For IL-12 p40/70, biotinylated Clone C17.8 was used; for IL-6, biotinylated Clone MP5–32C11 was used. Following another 2 hr of incubation, plates were washed, and 100 μL of diluted avidin peroxidase was added to each well (Zymed). After a 30-min incubation, plates were washed, and 100 μL of a solution containing 0.015% 3,3′,5,5′-tetramethylbenzidine hydrochloride (Sigma), 0.01% hydrogen peroxide in 0.1 M sodium citrate buffer, pH 4.0, was added. Plates were read at O.D. 380 on an automated microplate reader (Molecular

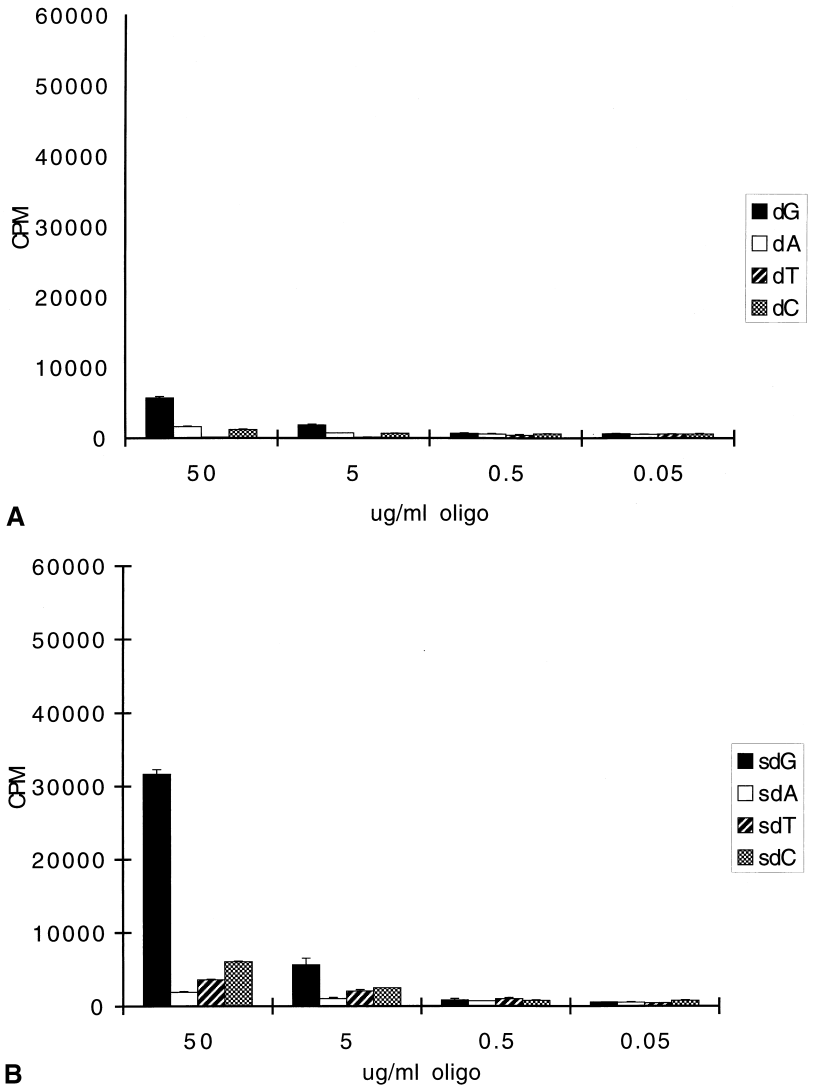

FIG. 1. Induction of proliferation by single base Po and Ps oligos. Spleen cells were stimulated by Po oligos (A) or Ps oligos (B) at the concentrations indicated. Proliferation was assessed by thymidine incorporation. Results are reported as mean counts ± SD of triplicate determinations.

Devices), and cytokine concentrations were derived from standard curves.

RESULTS

In these experiments, we compared the immunostimulatory activities of two sets of compounds synthesized as either Po or Ps derivatives. The first set of compounds were 30mers composed of a single base; the second set were 30mers composed of an ISS (AACGTT) flanked at 5′ and 3′ ends by 12 nucleotides of each base. Compounds with an ISS are denoted as AP signifying active palindrome, with the letter in parentheses indicating the flanking bases; "s" denotes phosphorothioate. We chose these compounds on the basis of prior studies indicating that flanking bases affect the magnitude of immune stimulation induced by Po oligos with these sequences [23, 24].

Figure 1 presents results of mitogenesis assays using the Po and Ps compounds consisting of a single base. As these data indicate, among Po oligos, a dG compound stimulated mitogenesis as shown by ^{3}H incorporation, whereas the other compounds had minimal activity. The sulfur substitution led to a similar pattern of stimulation among single base compounds, with the sdG [30] more active than the corresponding Po compound as well as other Ps compounds. In these experiments, the sdC [30] Ps oligo also induced limited mitogenesis as previously reported [19].

The impact of an ISS in the context of different flanking bases was tested next (Fig. 2). Among the Po oligos with an ISS, the AP(G) induced the greatest level of stimulation. This stimulation exceeded that of dG [30], indicating the activating effect of the ISS. The AP(C) and AP(A) induced more limited proliferation as previously reported [24]. In contrast, among Ps oligos of these sequences, all the compounds induced marked proliferation. Although the concentration–response curves of these compounds varied in terms of the concentration for optimal stimulation, at their peak, all four Ps compounds induced similar mitogenic responses. Comparison of data in Figs. 1 and 2 indicates the marked enhancement in immunostimulatory activities that results from both the presence of an ISS and the Ps backbone. Thus, the stimulation observed with dG [30]

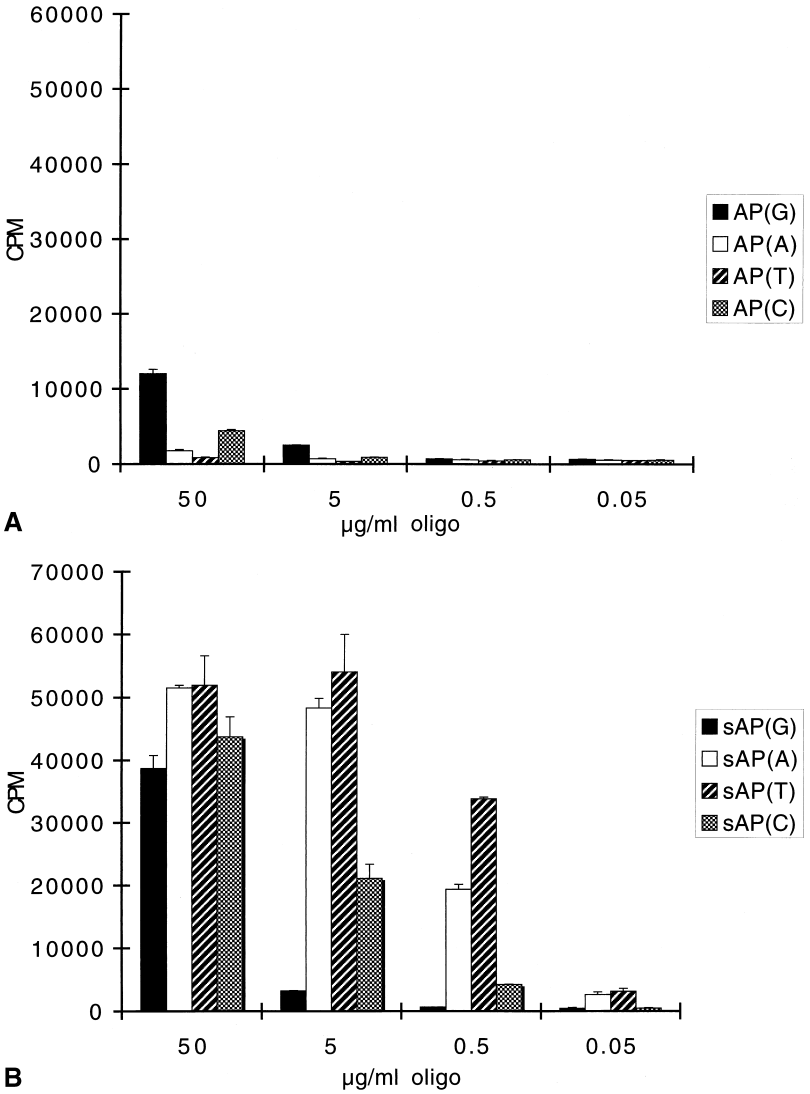

FIG. 2. Induction of proliferation by Po and Ps oligos containing an ISS. Spleen cells were stimulated with a series of Ps and Po oligos in which an ISS is flanked on the 5′ and 3′ ends by 12 residues of each base. AP indicates active palindrome, since the ISS can form a palindrome. Results are presented as in Fig. 1. A, Po compounds; B, Ps compounds.

could be achieved by sAP(A) and sAP(T) at approximately 100-fold lower concentrations.

The use of proliferation assays to assess cell activation by Po compounds can be complicated by the effects of pool dilutions from breakdown of these compounds into nucleotides [25, 26]. As an alternative assay of B cell activation, we therefore examined cell surface expression of the marker CD69. As previously shown, this marker can be used to measure activating effects of oligos [27]. Figure 3 presents results of this assay using the two sets of compounds. As these data indicate, among single base compounds, dG [30] was more active than the other oligos, similar to the results with the thymidine incorporation assays. Among Ps compounds of a single base, sdG [30] was also the most active.

Among compounds with an ISS, all the S-oligos with an ISS showed a high level of activity, which in all cases, was greater than that of the Po counterpart. Together, these results indicate that the extent of cell activation varies with backbone chemistry, with Ps compounds being more potent stimulators than Po compounds. Furthermore, the structure–function relationship for stimulation by Ps and Po compounds differed, with the enhancing effect of dG sequences present with Po compounds not observed with Ps compounds. Analysis of cell populations by double staining indicated that both B and T cells show an increase in CD69 (data not shown), although proliferation assays reflect B cell effects on the oligos [4].

To determine whether cytokine responses followed the same pattern as proliferation, the ability of the two sets of Po and Ps compounds to induce IL-6 and IL-12 was tested. Under the conditions of these assays, neither the Ps nor the Po compounds with single bases induced significant production of either IL-6 or IL-12 (data not shown), pointing to a requirement for an ISS for production of cytokines. For the IL-12 response, AP(G) and AP(T) compounds induced production of this cytokine at the highest concentration tested (Fig. 4). In contrast, S-oligos were active over a much larger range of concentrations, with sAP(A) inducing the highest response (Fig. 4). For the IL-6 response, Po compounds with an ISS failed to stimulate responses within the limits of detection (data not shown), while Ps oligos showed activity to 0.5 µg/mL. Similar to the IL-12 re-

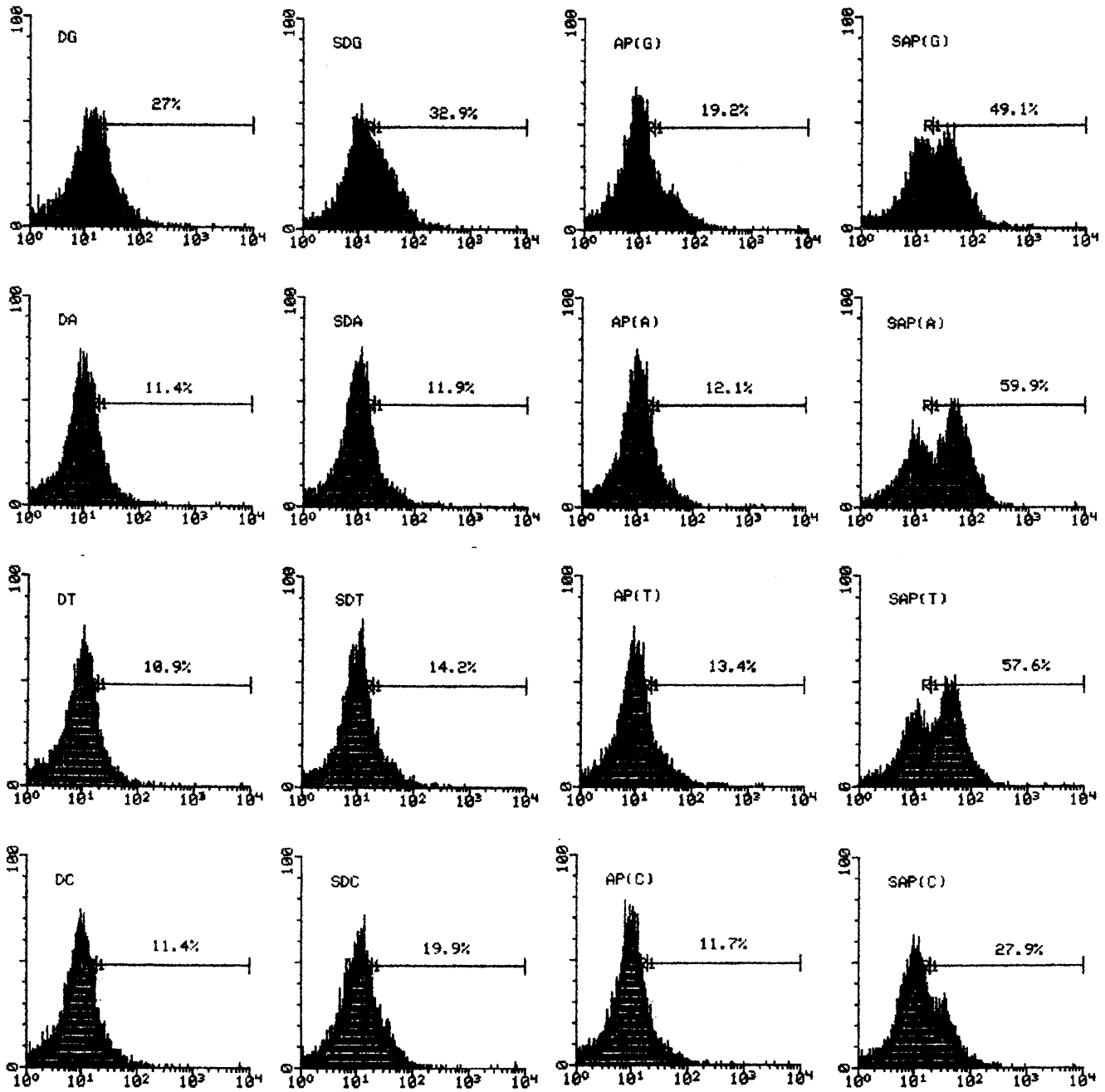

FIG. 3. Assessment of proliferation by cell surface CD69 expression. Induction of proliferation was assessed by cell surface CD69 expression by FACS. This figure presents a fluorescence profile of total spleen cells. The concentration of oligos in each culture was 50 μg/mL. The percentage of positive cells was defined using settings such that 90% of unstimulated control cells fell below the lower limit.

sponse, sAP(A) and sAP(T) were the most active (Fig. 5). Together, these results indicate that, while the Ps backbone increases immune stimulation by an oligonucleotide, the effect varies, depending on the sequence and response measured.

DISCUSSION

Results of these studies provide further insights into the structure–function relationships of immunostimulatory DNA. Importantly, these studies showed that Po and Ps oligos differ with respect to sequences leading to B cell activation as well as cytokine production. While, in general, Ps oligos were more active than Po oligos, the sequences causing optimum immune activation varied, depending on the response. Taken together with previous studies comparing activation of B cell responses by Ps and Po oligos, these observations indicate that immune stimulation by DNA depends on both base sequence and backbone chemistry [19, 21, 22, 28, 29].

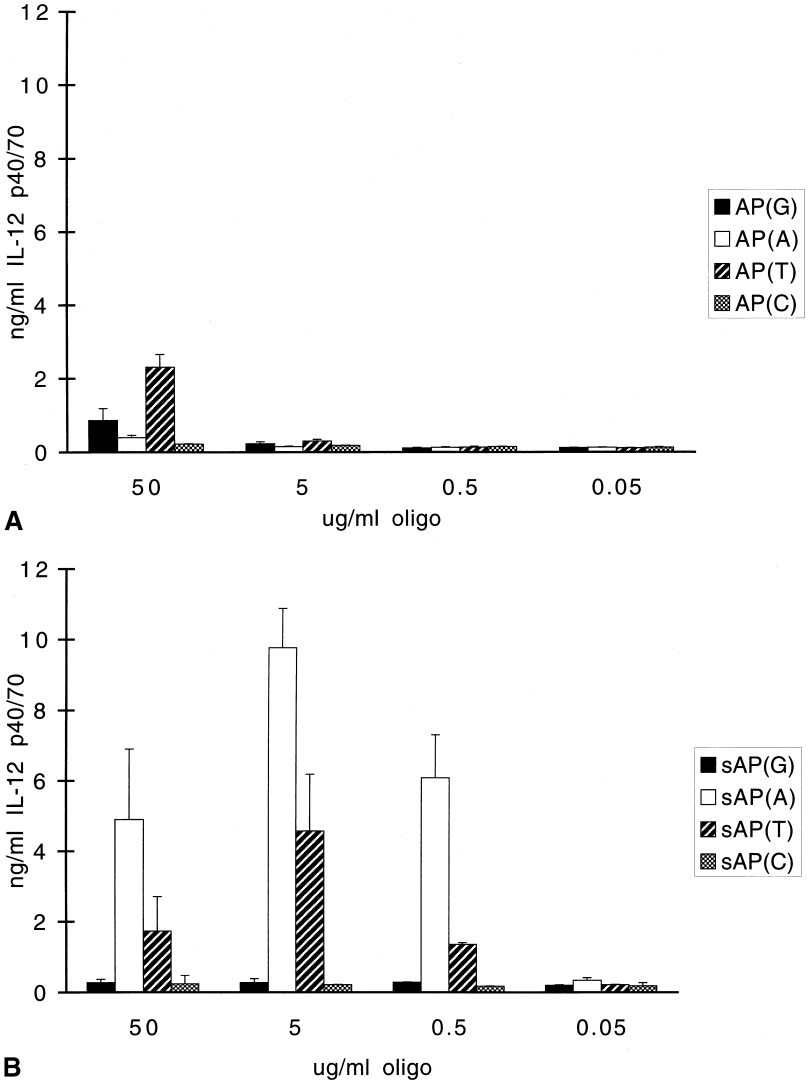

FIG. 4. Stimulation of IL-12 production by Po and Ps oligos. Po and Ps oligos containing an ISS were used to stimulate spleen cell cultures as described in Materials and Methods. IL-12 p40/70 levels were measured by ELISA. Results are reported as means ± SD of triplicate cultures. A, Po compounds; B, Ps compounds.

In these studies, we used a series of compounds that consisted of either a single base or an ISS embedded in runs of 12 nucleotides of each base. We investigated these compounds because of evidence that, while CpG motifs lead to immune stimulation, other sequences may either stimulate immune responses by themselves or enhance

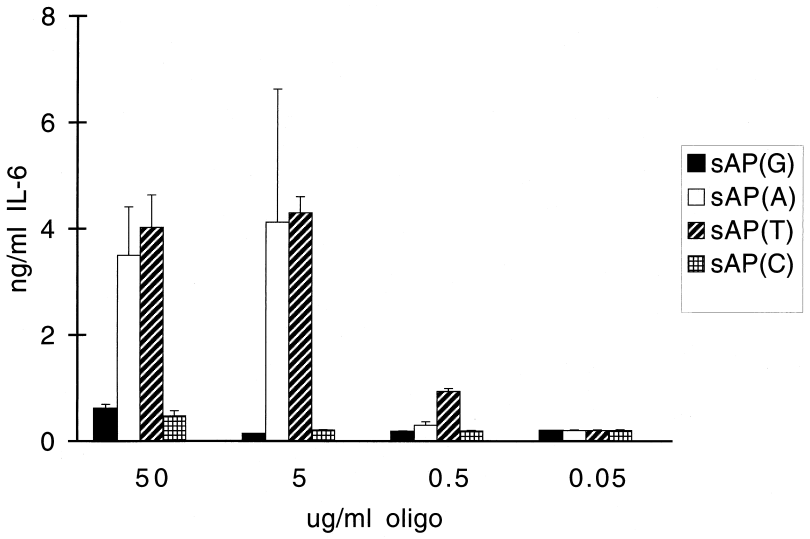

FIG. 5. Stimulation of IL-6 production by Ps oligos. Spleen cells were stimulated with various concentrations of Ps oligos, and IL-6 levels were measured in supernatants by ELISA. Results are reported as means ± SD of triplicate cultures.

responses induced by an ISS. Thus, among single base compounds, dG oligos as phosphodiesters can stimulate murine B cell activation [19]. Furthermore, while dG sequences alone fail to induce cytokine production, their presence leads to a dramatic increase in the ability of an ISS to stimulate IFN production and natural killer cell activation [30]. The mechanisms for dG induction of mitogenesis are not understood. The influence of dG on cytokine production, however, has been attributed to enhanced cell uptake via the Type A macrophage scavenger receptor (MSR). This receptor can bind a variety of polyanions but appears to have a preference for dG sequences among nucleic acids [31–33].

In the studies presented herein, we assessed cell activation by both thymidine incorporation and cell surface CD69 expression. In culture, Po oligos are subject to nuclease digestion, leading to release of nucleotides that can dilute the thymidine pool. For compounds containing thymidines, especially at the 3′ end, the effect of pool dilution prevents detection of a proliferative response by thymidine incorporation [25, 26]. For Ps oligos, however, effects of pool dilution are negated because of the stability of these compounds in culture. With both assays of cell activation, Ps oligos displayed different apparent structure–function relationships than Po compounds. Thus, whereas dG flanks produced the greatest stimulation of proliferation among Po compounds with an ISS, flanks of any of the 4 bases produced similar levels of stimulation with the Ps compounds. These findings support observations of Monteith *et al.* [22], indicating that immune stimulation by phosphorothioates is a class effect of these compounds that is not related solely to the content of ISS.

The analysis of production of IL-6 and IL-12 provides further evidence that, while immune stimulation reflects both sequence and backbone chemistry, the optimal sequence may vary, depending upon response. Thus, among Ps compounds with an ISS, a compound with dA flanks produced the highest stimulation for IL-12, whereas compounds with dA and dT flanks produced the highest stimulation of IL-6; for both responses, dG and dC flanks led to only limited responses. The variable stimulation of cytokines by Ps oligos contrasts with their uniform enhancement of mitogenesis observed with compounds with a Ps backbone. Our findings on selective stimulation of cytokines by oligos is supported by Lipford *et al.* [34], who described an oligo called IL-12-P-40 that could induce IL-12 without inducing TNF-α.

In considering the possibility that oligos can stimulate immune responses selectively, it is important to note that other studies on this issue have suggested that the relative potency of oligos in the induction of cytokines and mitogenesis is similar [9]. The differences among studies in the relationship of sequence to stimulation may arise from a number of variables, including the length and sequence of the compounds, the source of cells (e.g. cultured cell lines vs fresh tissue preparations), as well as administration of oligos *in vivo* and *in vitro*. In this regard, the cell type responsible for cytokine production may differ depending on the experimental system. Both B cells and monocytes/macrophages can be a source of IL-12 production induced by oligonucleotides. In some studies, B cells have been identified as the IL-12 producing cell type, whereas in others, macrophages/monocytes appear to be the predominant source [35].

While the basis of immune stimulation by oligos requires further analysis, our findings raise several important issues. The first issue concerns the relationship between immune activation by Ps and Po oligos. As shown here and in other studies, Ps and Po compounds differ in their structure–function relationship, possible mode of immune activation (i.e. requirement for cellular uptake), and pattern of interaction with DNA binding proteins. These considerations suggest that, while Ps oligos may have promise as immunomodulatory reagents, their immune properties may differ from Po oligos, leading to caution in their use as models of immune activity of bacterial DNA.

A second issue raised by our studies concerns the potential to design oligos with specific functions. Thus, among compounds studied, we have found examples of a compound [e.g. sdG [30]] that can stimulate B cells without effects on cytokines; in addition, we have identified compounds that can stimulate B cells while inducing either high or low amounts of IL-6 and IL-12. Each of these compounds could find specific use as an immunomodulator. In this regard, DNA can stimulate other responses (e.g. MIP-1β and MCP-1) that may also vary with respect to sequence requirements [36]. Until these activities have been analyzed comprehensively, the full potential for "designer" oligos is speculative.

A final issue relates to the influence of backbone chemistry on immune activation. Whereas the Po oligo AP(A) had low activity in proliferation as well as cytokine assays, sAP(A) caused the most potent stimulation of IL-6 and was highly mitogenic. In the future design of oligos, it will be important to determine whether the differences between Ps or Po compounds relates to their relative content of stimulatory and inhibitory sequences. In this regard, previous studies have shown that phosphorothioate dG oligos can inhibit IFN-γ production stimulated by concanavalin A or phorbol 12-myristate 13-acetate and ionomycin [37]. The low stimulation of IL-12 and IL-6 by the sAP(G) compound may, in fact, represent opposing effects of ISS and inhibitory sequences (dG). Studies are in progress to address these issues, focusing on the role of DNA sequence in the responses of B cells and macrophages.

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