Activation of Human B Cells by Phosphorothioate Oligodeoxynucleotides

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Abstract

To investigate the potential of DNA to elicit immune responses in man, we examined the capacity of a variety of oligodeoxynucleotides (ODNs) to stimulate highly purified T cell–depleted human peripheral blood B cells. Among 47 ODNs of various sequences tested, 12 phosphorothioate oligodeoxynucleotides (sODNs) induced marked B cell proliferation and Ig production. IL-2 augmented both proliferation and production of IgM, IgG, and IgA, as well as IgM anti-DNA antibodies, but was not necessary for B cell stimulation. Similarly, T cells enhanced stimulation, but were not necessary for B cell activation. After stimulation with the active sODNs, more than 95% of B cells expressed CD25 and CD86. In addition, B cells stimulated with sODNs expressed all six of the major immunoglobulin V_H gene families. These results indicate that the human B cell response to sODN is polyclonal. Active sODN coupled to Sepharose beads stimulated B cells as effectively as the free sODN, suggesting that stimulation resulted from engagement of surface receptors. These data indicate that sODNs can directly induce polyclonal activation of human B cells in a T cell–independent manner by engaging as yet unknown B cell surface receptors. (*J. Clin. Invest.* 1996. 98:1119–1129.) Key words: oligonucleotides • B cells • autoimmunity • antisense • antibody

Introduction

DNA is a complex macromolecule whose immunological activities are influenced by its base composition and base modification, as well as helical orientation (1, 2). Certain unusual DNA structures (e.g., Z-DNA) can induce significant antibody responses when administered to normal mice (1–5). In addition, bacterial DNA, as well as certain synthetic oligodeoxynucleotides (ODNs)[1] containing an unmethylated CpG motif, can induce proliferation and Ig production by murine B cells. In contrast, mammalian DNA does not stimulate B cell responses (6–8). These results indicate that bacterial DNA and ODNs of certain structures can activate murine B cells, although the mechanism is still unclear.

The immunostimulatory activity of ODNs has also been demonstrated with antisense compounds synthesized as phosphorothioate oligodeoxynucleotides (sODNs). Those compounds have a sulfur substitution for a nonbridging oxygen in the backbone and differ from phosphodiesters in a number of properties, such as nuclease resistance and melting temperature. Among 18–27-bp sODNs designed to inhibit the expression of specific target genes, some induced activation of murine lymphocytes (9–11). One of the most stimulatory antisense sODNs is complementary to a portion of the rev region of the HIV genome. This antisense compound induced marked proliferation and immunoglobulin production by murine B cells in vitro. Moreover, mice developed massive splenomegaly and polyclonal hypergammaglobulinemia within 2 d after intravenous injection of this sODN (9). B cell stimulation by the antisense sODN does not appear to result from inhibition of specific genes by the compound, but rather results from direct activation of B cells, although the sequence(s) and structure(s) accounting for this phenomenon remain unknown.

The immunological properties of DNA have also been investigated in the context of autoimmune disease, especially systemic lupus erythematosus (SLE). Although anti-DNA antibodies have features of antigen selection, including evidence of somatic mutation, the nature of the driving antigen and the contribution of polyclonal activation of B cells to the initiation of this response remain unclear. It has been proposed, however, that stimulation of B cells by either bacterial DNA or the DNA in the plasma of patients with active SLE may contribute to anti-DNA antibody production (6, 12–16).

Although bacterial DNA and certain ODNs can stimulate murine B cells, much less information is available on the immunostimulatory capacity of these materials for human B cells. Because of the differences in the responsiveness of human and murine B cells to certain stimuli, it is not possible to extrapolate results obtained from mouse to man. Therefore, the current experiments were undertaken to determine whether ODNs or bacterial DNA could directly stimulate human B cells. To assess their activities, a panel of synthetic ODNs was examined for their ability to stimulate highly purified human peripheral blood B cells. Of 47 phosphorothioates and phosphodiesters tested, 12 sODNs stimulated marked polyclonal activation of human B cells in a T cell–independent manner. B cell activation appeared to result from engagement of an as yet unknown surface receptor(s). These results thus document the novel mitogenic properties of certain sODNs for

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1. *Abbreviations used in this paper:* ODN, oligodeoxynucleotide; PE, phycoerythrin; SLE, systemic lupus erythematosus; sODN, phosphorothioate derivative of ODN.

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human B cells and provide the basis for understanding their capacity to function as polyclonal B cell activators.

Methods

DNA and ODNs. 47 ODNs varying in length from 5 to 30 bp, including both phosphorothioate and phosphodiester derivatives, were synthesized using standard phosphoramidite chemistry methods (17) and purchased from The Midland Certified Reagent Co. (Midland, TX). The sequence, size, and a brief description of the ODNs are shown in Table I and later in Table III. *Escherichia coli* DNA and mammalian DNA were kindly provided by Dr. Arthur Krieg (University of Iowa Medical Center, Iowa City, IA) and prepared as described (6).

Cell preparation and purification. PBMCs were isolated from heparinized blood of healthy adult volunteers by centrifugation over sodium diatrizoate/ficoll gradients (Sigma Chemical Co., St. Louis, MO). PBMCs were depleted of NK cells and monocytes by incubation with 5 mM L-leucine methylester (Sigma Chemical Co.) in serum-free RPMI 1640 (BioWhittaker, Inc., Walkersville, MD) (18–20). After washing, the B cells were separated from T cells by two cycles of rosetting with neuraminidase-treated sheep RBC (21). The resultant B cells were analyzed by flow cytometry and found to be $> 90\%$ $CD19^+$ by using phycoerythrin (PE)-conjugated anti–CD19 mAb (Sigma Chemical Co.) and $< 1\%$ $CD3^+$ by using anti–CD3 mAb (American Type Culture Collection, Rockville, MD) and FITC-labeled $F(ab)'_2$ sheep anti–mouse IgG (Calbiochem-Novabiochem Corp., La Jolla, CA). In some experiments, $CD19^+$ B cells were purified with a

Table I. Sequence, Size, and Description of 34 ODNs

Cmpd	Sequence of ODNs	Size	Description
		base pairs	
Phosphorothioates:			
1082	5′-GCCGAGGTCCATGTCGTACGA-3′	21	Antisense to a translation initiation condon region within the UL13 open reading frame of HSV (10)
2105	5′-TTGCTTCCATCTTCCTCGTC-3′	20	
DSP13	5′-GGGGGGGGGG-3′	10	
DSP14	5′pGGGGGGGGGGGGGGGGGGGG-3′	20	5′ Phosphorothioate
DSP16	5′AAAAAAAAAAAAAAAAAAAA-3′	20	
DSP19	5′-NNNNNNNNNNNNNNNNNNN-3′	20	Randomer N = ACGT
DSP20	5′-NNNNNNNNNN-3′	10	Randomer N = ACGT
DSP25	5′-GGGGGGGGGGGGGGGGGGGG-3′	20	
DSP26	5′-CCCCCCCCCCCCCCCCCCCC-3′	20	
DSP27	5′-GGGGG-3′	5	
DSP28	5′-TTTTTTTTTTTTTTTTTTTT-3′	20	
DSP30	5′-TCGTCGCTGTCTCCGCTTCTTCTTGCC-3′	27	Antisense to rev gene of HIV (9)
DSP31	5′-AAAAAAGG-3′	8	
DSP34	5′-AAAAAAAG-3′	8	
DSP35	5′-CCCCCCGG-3′	8	
DSP36	5′-GGGGGGGGGGGGGGGG-3′	15	
DSP37	5′-AAAAAAAAA-3′	9	
DSP39	5′-TTTTTTTTT-3′	9	
DSP40	5′-TTTTTTTTTT-3′	10	
DSP42	5′-TCGTCGCTGTCTCCG-3′	15	Truncated 5′ portion of DSP30
DSP43	5′-CTGTCTCCGCTTCTT-3′	15	Truncated central portion of DSP30
DSP44	5′-CCGCTTCTTCTTGCC-3′	15	Truncated 3′ portion of DSP30
DSP49	5′-TCGTCGTCGTCGTCG-3′	15	$(TCG)_5$
DSP50	5′-TCGTCGGGGGGGGGG-3′	15	$(TCG)_2$ of poly G
DSP54	5′-ACGACGACGACGACG-3′	15	$(ACG)_5$
DSP55	5′-TCCTCCTCCTCCTCC-3′	15	$(TCC)_5$
DSP56	5′-TGGTGGTGGTGGTGG-3′	15	$(TGG)_5$
DSP57	5′-TAGTAGTAGTAGTAG-3′	15	$(TAG)_5$
Phosphodiesters			
DSP17	5′-GCCGAGGTCCATGTCGTACGC-3′	21	Diester of 1082
DSP18	5′-NNNNNNNNNNNNNNNNNNN-3′	20	Randomer N = ACGT
DSP21	5′-GGGGGGGGGGGGACCGGTGGGGGGGGGGGG-3′	30	Palindromic sequence that did not enhance NK cell activity (38, 39)
DSP22	5′-GGGGGGGGGGGGAACGTTGGGGGGGGGGGG-3′	30	Palindromic sequence that enhances NK cell activity (38, 39)
DSP23	5′-GGGGGGGGGGGGGGGGGGGGG-3′	20	
DSP24	5′-CCCCCCCCCCCCCCCCCCCC-3′	20	

Cmpd, compound.

CEPRATE LC kit (CellPro, Inc., Bothell, WA) following the manufacturer's protocol.

Sedimented rosete-forming cells from the first centrifugation were treated with isotonic NH_4Cl to lyse the sheep RBC and purified on a nylon-wool column. T cell populations were > 95% $CD3^+$ and were subjected to mitomycin C treatment (40 μg/ml) as previously described (22).

Culture conditions. B cells (2.5–5 × 10^4/well) were cultured with ODNs in U-bottom 96-well microtiter plates (Costar, Corp., Cambridge, MA) alone or in the presence of IL-2 (50 U/ml) (Hoffman-La Roche, Nutley, NJ) or mitomycin C–treated T cells. All cultures were conducted in RPMI 1640 medium supplemented with penicillin G (200 U/ml), gentamicin (10 μg/ml), L-glutamine (0.3 μg/ml), and 10% FBS (Life Technologies, Inc., Grand Island, NY).

Assay of B cell DNA synthesis. The B cells (5 × 10^4/well) were incubated in triplicate for 4 d at 37°C with 1 μCi ^{3}H-labeled TdR (6.7 Ci/mM; ICN Biomedicals, Inc., Irvine, CA) present for the last 18 h. The cells were harvested onto glass filter paper, and ^{3}H-labeled TdR incorporation was determined by liquid scintillation spectroscopy.

Analysis of Ig secretion. The B cells (2.5 × 10^4/well) were incubated in duplicate for 14 d at 37°C, after which supernatants were collected from the cultures and frozen until analysis. Immunoglobulin in the culture supernatants was quantitated by using isotype-specific ELISA, as previously described (23, 24).

Flow cytometric analysis. To analyze the expression of CD86 and CD25, ~ 5 × 10^5 B cells were collected from culture wells and stained with either PE-conjugated anti–CD86 or anti–CD25 or appropriate control monoclonal antibodies for 30 min at 4°C. After washing to remove unbound mAbs, cells were resuspended and analyzed by flow cytometry with the use of the FACScan® system (Becton Dickinson & Co., Mountain View, CA). Anti–CD25 is a PE-conjugated mouse IgG1 mAb against the α chain of the IL-2R (Becton Dickinson & Co.). Anti–CD86 or B70 (B7-2) is a PE-conjugated mouse mAb against the human B cell activation marker CD86 (B70). IgG2b kappa is the control mAb for B70 (Pharmingen, San Diego, CA). 64.1 is an IgG2a mAb directed at the CD3 molecular complex on mature T cells (25).

RNA extraction. B cells were incubated in the same culture conditions previously used for analysis of Ig secretion. The contents of 60 multiple microwells were pooled and harvested. Total RNA was extracted by using the RNeasy method, following the manufacturer's protocol (QIAGEN Inc., Chatsworth, CA).

cDNA synthesis and PCR analysis. After quantification of isolated RNA by spectrophotometry, cDNA was synthesized by priming with 500 ng oligo dT (Pharmacia LKB Biotechnology Inc., Almeda, CA) in Ultraspec diethylpyrocarbonate water (Biotecx Laboratories, Inc., Houston, TX), 5 mM DTT, 0.5 mM dNTP, 5 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$ using 200 U Superscript reverse transcriptase (Life Technologies, Inc.) per 1 μg of total RNA.

Each PCR reaction was performed with 1 μg of cDNA in an 80-μl volume containing Ultraspec water (Cinna/Biotecx, Friendswood, TX), 50 mM KCl, 10 mM Tris-Cl (pH 9.0), 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.9 μM of each primer, and 2.5 U of Taq polymerase (Promega Corp., Madison, WI). The 5′ primers corresponding to each of the leader sequences of the six V_H families are: V_H1, 5′-CCATGGACTGGACCTGGA-3′; V_H2, 5′-ATGGACATACTTTGTTCCAC-3′; V_H3, 5′-CCATGGAGTTTGGGCTGAGC-3′; V_H4 5′-ATGAAACACCTGTGGTTCTT-3′; V_H5, 5′-ATGGGGTCAACCGCCATCCT-3′; and V_H6, 5′-ATGTCTGTCTCCTTCCTCAT-3′. The 3′ primer for the heavy chain of the IgM constant region is 5′-GGAATTCTCACAGGAGCCACG-3′. Amplification consisted of a 5-min denaturing step at 94°C followed by 35 cycles of amplification at the following conditions: 1 min of denaturation at 94°C, 1 min of annealing at the indicated temperatures (54°C for V_H1 and V_H6, 58°C for V_H3 and V_H5, and 50°C for V_H2 and V_H4), and 1 min of extension at 72°C. PCR products were resolved by electrophoresis on a 1% agarose gel in 0.5× Tris boric acid EDTA. After denaturation of the gel in 1.5 M NaCl and 0.5 M NaOH for 30 min, the PCR products were transferred onto a nylon membrane (Bio-Rad Laboratories, Hercules, CA) using a vacuum-blotting apparatus. The membrane was baked at 80°C for 90 min before screening with a ^{32}P 5′ end-labeled probe, specific for PCR products amplified with a given primer pair. Hybridization was conducted in Rapid-hyb buffer (Amersham LIFE SCIENCE, Little Chalfont, UK) at 42°C. Blots were washed in 0.1× SSC and 0.1% SDS at 45°C for 50 min and subjected to autoradiography.

Assay of anti-DNA antibodies. Polystyrene microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with calf thymus DNA (15 μg/ml) or ODNs (5 μg/ml except 1 μg/ml for DSP30) diluted in Tris buffer (pH 9.4) by incubating them overnight at 4°C. Plates were then washed with PBS and blocked with 2% nonfat dry milk. The plates were then washed with 1 M NaCl 5% Tween and incubated with appropriately diluted culture supernatants or PBS for 2 h at room temperature. The washed plates were then incubated with peroxidase-conjugated, affinity-purified goat antibody (Ab) to human μchain (Biosource International, Camarillo, CA). After addition of substrate (TMB; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), the development of colored reaction product was quantitated with an ELISA reader (Biokinetics reader EL312; Bio-Tek Instruments, Inc., Winooski, VT).

Techniques of coupling Sepharose beads with ODNs. ODNs were synthesized with a lysine residue linked to the 5′ end as described (17). CNBr-activated Sepharose 4B beads were coated with modified ODNs according to the manufacturer's direction (Pharmacia LKB Biotechnology Inc.). Briefly, modified ODNs were dissolved in coupling buffer (0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl) and incubated overnight at 4°C with constant rotating with an appropriate amount of Sepharose beads that had been swollen and washed in 1 mM HCl (pH 2–3). After washing with coupling buffer and blocking by incubation with 0.1 M glycine at room temperature for 2 h, the beads were washed with three cycles of alternating pH buffers (coupling buffer, pH 8.3, and acetate buffer, pH 4.0). The beads were then resuspended and stored in PBS. Control beads were coupled with glycine only.

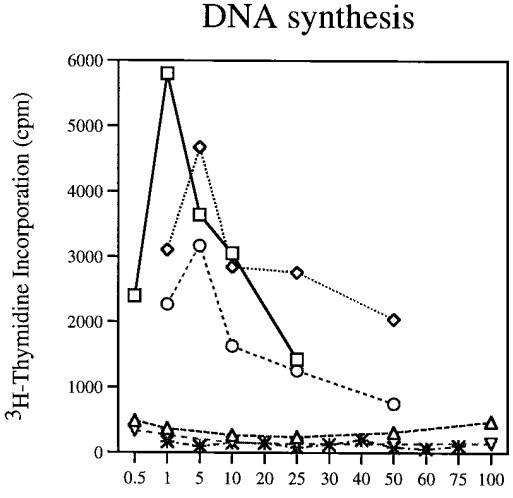


Figure 1. B cell proliferation induced by DNA and sODNs. B cells (50 × 10^3/well) were cultured with various concentrations of DNA (0.5–100 μg/ml) or ODNs (1–50 μg/ml except 0.5–25 μg/ml for DSP30 and 1–75 μg/ml for DSP17) for 4 d. Proliferation was assessed by [^{3}H]thymidine incorporation. The data are the mean of triplicate cultures with an SEM of < 10% and are representative of three separate experiments, each carried out with B cells from a different donor. Control B cells cultured in medium alone incorporated 123 cpm of [^{3}H]thymidine. □, DSP30; ◇, 1082; ○, 2105; △, *E. coli* DNA; ▽, mammalian DNA; *, DSP17.

Cmpd	SEQUENCE OF OLIGODEOXYNUCLEOTIDE	size
PHOSPHOROTHIOATES:		
1082	5′-GCC GAG GTC CAT GTC GTA CGC-3′	21bp
2105	5′-TTG CTT CCA TCT TCC TCG TC-3′	20bp
DSP30	5′-TCG TCG CTG TCT CCG CTT CTT CTT GCC-3′	27bp
DSP39	5′-TTT TTT TTT-3′	9bp
DSP40	5′-TTT TTT TTT T-3′	10bp
DSP28	5′-TTT TTT TTT TTT TTT TTT TT-3′	20bp
DSP37	5′-AAA AAA AAA A-3′	9bp
DSP16	5′-AAA AAA AAA AAA AAA AAA AA-3′	20bp
DSP27	5′-GGG GG-3′	5bp
DSP13	5′-GGG GGG GGG G-3′	10bp
DSP36	5′-GGG GGG GGG GGG GGG-3′	15bp
DSP25	5′-GGG GGG GGG GGG GGG GGG GG-3′	20bp
DSP14	5′p-GGG GGG GGG GGG GGG GGG GG-3′	20bp
DSP26	5′-CCC CCC CCC CCC CCC CCC CC-3′	20bp
DSP20	5′-NNN NNN NNN N-3′	10bp
DSP19	5′-NNN NNN NNN NNN NNN NNN NN-3′	20bp
DSP31	5′-AAA AAA GG-3′	8bp
DSP34	5′-AAA AAA AG-3′	8bp
DSP35	5′-CCC CCC GG-3′	8bp
DIESTERS:		
DSP17	5′-GCC GAG GTC CAT GTC GTA CGC-3′	21bp
DSP18	5′-NNN NNN NNN NNN NNN NNN NN-3′	20bp
DSP21	5′-GGG GGG GGG GGG ACC GGT GGG GGG GGG GGG-3′	30bp
DSP22	5′-GGG GGG GGG GGG AAC GTT GGG GGG GGG GGG-3′	30bp
DSP23	5′-GGG GGG GGG GGG GGG GGG GG-3′	20bp
DSP24	5′-CCC CCC CCC CCC CCC CCC CC-3′	20bp
No stimulus		

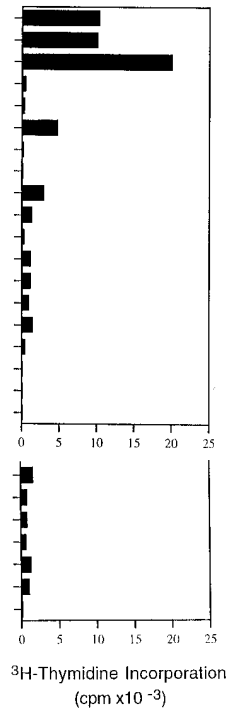


Figure 2. B cell proliferation induced by sODNs. B cells (50×10^3/well) were cultured with various concentrations of ODNs (1–50 μg/ml) for 4 d and proliferation was assessed by [^{3}H]thymidine incorporation. The data indicate the results with the optimal stimulatory concentrations, usually 5 μg/ml (except 1 μg/ml for DSP30 and 25 μg/ml for DSP20). Data with DSP17 were obtained by incubation of B cells with 5 μg/ml DSP17, and then adding 5 μg/ml of DSP17 to culture daily. A single addition of DSP17 induced no response. The data are the mean of triplicate cultures with an SEM of < 10% and are representative of three separate experiments, each carried out with B cells from a different donor. Cmpd, compound.

Results

Specific ODNs induce B cell proliferation and Ig production. The initial experiments examined whether bacterial DNA and various ODNs could induce human B cell proliferation and Ig production. Highly purified human B cells were cultured with various concentrations of DNA (0.5–100 μg/ml) or the ODNs listed in Table I (1 μg/ml–50 μg/ml) and [^{3}H]thymidine incorporation was measured. As shown in Fig. 1, three of the phosphorothioate ODNs (1082, 2105, and DSP30) consistently induced significant B cell proliferation, whereas neither bacterial DNA nor mammalian DNA was stimulatory (optimal stimulatory concentrations: 1 μg/ml for DSP30 and 5 μg/ml for 1082 and 2105). The other phosphorothioates (Figs. 1 and 2) induced minimal but nonetheless detectable responses that were statistically different than [^{3}H]thymidine incorporation manifested by B cells cultured alone. Of note, DSP17 (the diester form of 1082) was much less stimulatory within this concentration range even when added to culture repetitively (mean maximum response to DSP17 = 615±22 cpm vs 3,113±93 for 1082). Although modest, the response induced by DSP17 was significantly greater than thymidine incorporation of control B cells (99±7, $P < 0.01$). It should be noted that similar results were noted with B cells obtained from 12 normal donors.

The sODNs that induced maximal proliferation also induced Ig production from purified B cells. (Fig. 3). Thus, 1082, 2105, and DSP30 (first three columns) stimulated limited production of IgM, IgG, and IgA. Of note, however, the sODNs induced Ig production from highly purified B cells in the absence of exogenous cytokines. The other ODNs induced little Ig production. Similar results were noted with B cells obtained from eight normal donors.

Increased expression of activation markers induced by sODNs. To assess the effects of sODNs on the expression of activation markers, B cells were cultured with or without sODNs for 48 h and then examined for expression of CD86 (B7-2) and CD25 (IL-2R). As can be seen in Fig. 4, resting B cells express little CD25 and CD86. However, after stimulation by 1082, 2105, and DSP30, > 95% of B cells expressed CD86 and CD25. sODNs that induced minimal proliferation also caused either minimal or no increased expression of CD86 and CD25. Results obtained with one of these nonstimulatory compounds (DSP25) is shown in Fig. 4. The finding that

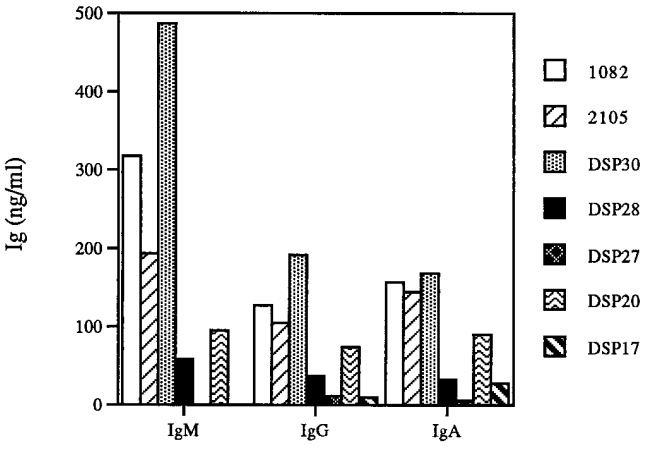


Figure 3. Ig production induced by sODNs. B cells (25×10^3/well) were cultured with ODNs at their optimal stimulatory concentrations of 5 μg/ml (1 μg/ml for DSP30 and 25 μg/ml for DSP20). Supernatants were harvested on day 14 and Ig production was assessed by ELISA. The data presented are the mean of duplicate cultures and are representative of four separate experiments, each carried out with B cells from a different donor. Control cells cultured in medium alone produced < 10 ng/ml of Ig. B cells cultured with *E. coli* DNA or mammalian DNA at 50 μg/ml for 14 d also produced < 10 ng/ml of Ig.

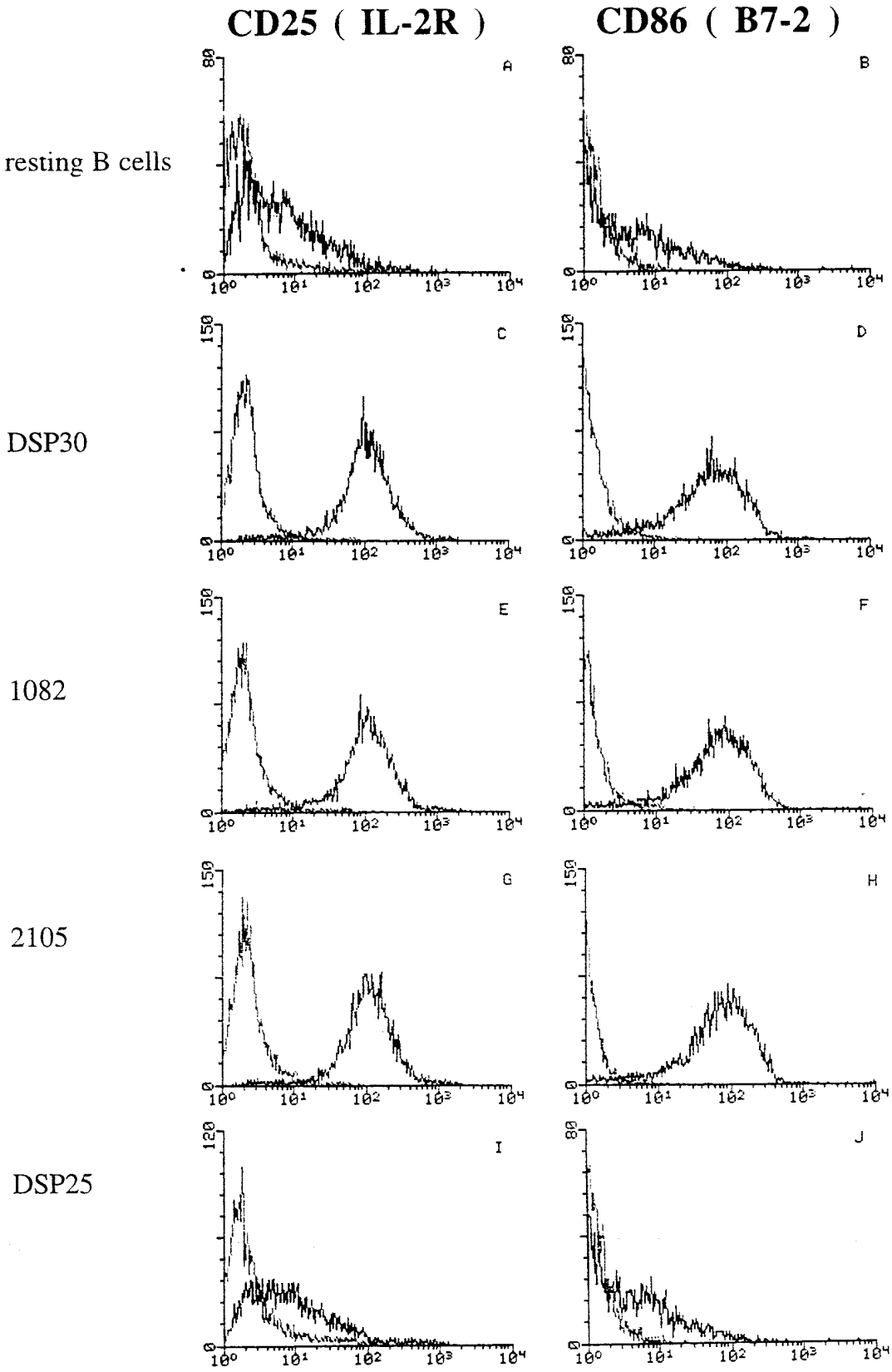


Figure 4. sODNs induce increased expression of CD86 and CD25. B cells (2×10^5/well) were cultured for 48 h with medium alone (*A* and *B*), DSP30 (*C* and *D*), 1082 (*E* and *F*), 2105 (*G* and *H*) or DSP25 (*I* and *J*) at their optimal concentrations (1 μg/ml for DSP30 and 5 μg/ml for the others). 5×10^5 B cells were collected from each culture and stained with mAbs against CD86 (*B7-2*) (black) or CD25 (*IL-2R*) (black) or isotype-matched control mAbs (gray). The FACS® analysis data presented are representative of five separate experiments, each carried out with B cells from a different donor.

sODNs induced activation marker expression by > 95% of B cells implies that the response is polyclonal.

B cell responses to sODNs are enhanced by IL-2. To investigate the effect of IL-2 on B cell activation induced by the sODNs, B cells were cultured with ODNs in the presence or absence of IL-2 and analyzed for proliferation and Ig production (Fig. 5). IL-2 markedly enhanced responses of B cells activated by the most stimulatory sODNs. However, IL-2 did not

enhance responses by B cells stimulated by ODNs that induced minimal proliferation. In the absence of ODNs, B cells did not proliferate or secrete Ig with IL-2 alone. These results indicate that IL-2 enhanced responses to stimulatory ODNs, but not to the others.

B cell responses to sODNs are augmented by T cells that do not proliferate in response to sODNs. The ability of T cells to enhance B cell responses to sODNs was examined next (Fig. 6). Coculture of B cells and mitomycin C–treated T cells in the presense of most stimulatory sODNs enhanced both B cell proliferation and Ig production. The degree of enhancement was proportionate to the number of T cells. No proliferation or Ig production was detected in cultures of T and B cells in the absence of sODNs. Similarly, the sODNs did not directly stimulate proliferation of control T cells that had not been treated with mitomycin C (T alone). Altering the concentration of sODNs from 1 to 25 μg/ml likewise did not induce T cell proliferation (data not shown). Thus, T cells as well as IL-2 enhance B cell responses to maximally stimulatory sODNs, whereas the sODNs do not directly induce T cell proliferation.

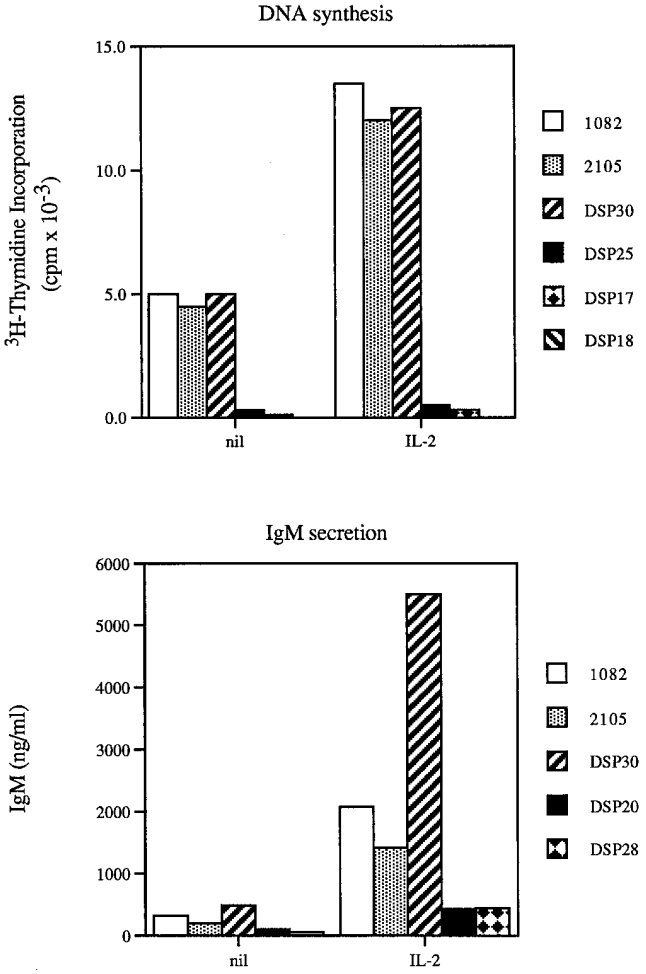
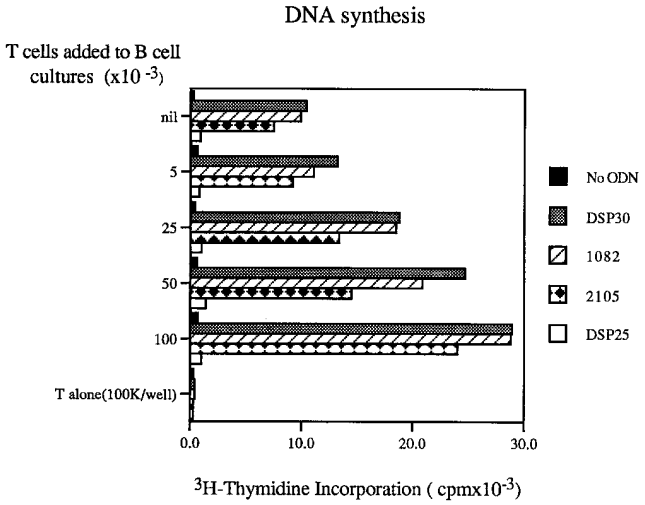


Figure 5. IL-2 enhances B cell responses induced by sODNs. B cells were cultured with ODNs at their optimal concentrations (1 μg/ml for DSP30, 25 μg/ml for DSP20, and 5 μg/ml for others) in the presence or absence of IL-2 (50 U/ml). Proliferation and Ig production were assessed as described. Responses of B cells alone or B cells cultured with IL-2 were < 100 cpm of [^{3}H]thymidine incorporation and < 10 ng/ml of Ig produced. The data presented are representative of three experiments, each carried out with B cells from an individual donor.



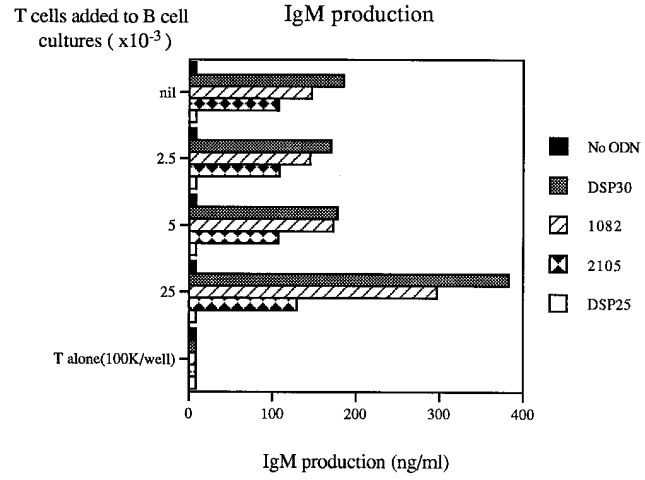


Figure 6. T cells augment B cell responses induced by sODNs. B cells (25×10^3/well) were cocultured with different numbers of mitomycin C–treated T cells ($2.5–100 \times 10^3$/well) in the presence or absence of sODNs at their optimal concentrations (1 μg/ml for DSP30 and 5 μg/ml for others). Proliferation and IgM production were measured as described. The results of one of three experiments, each carried out with cells obtained from a different donor, with similar findings are shown. When T cells alone were tested (*T alone*), they were not treated with mitomycin C, but were cultured (100×10^3/well) with sODNs at concentrations from 1 to 25 μg/ml. Data indicated proliferation induced by the concentration (5 μg/ml) that induced the maximal response.

B cell activation induced by sODNs is polyclonal. To confirm that B cell activation induced by sODNs is polyclonal, IgM-producing B cells were analyzed for the usage of heavy chain variable region genes after culture of B cells with DSP30 and IL-2 for 8 d. RNA was harvested and analyzed by RT-PCR using μchain and V_H-specific primers. As can be seen in Fig. 7, little V_H mRNA was detected in B cells cultured with IL-2 alone. However, B cells stimulated with DSP30 and IL-2 contained

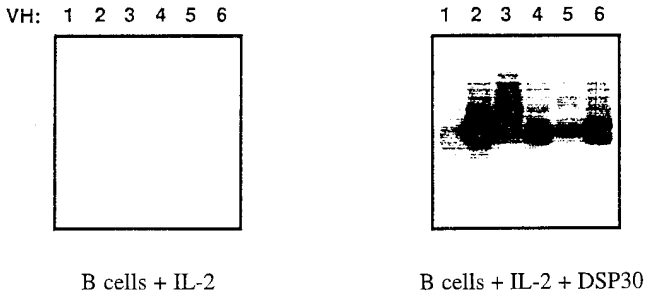


Figure 7. Polyclonal activation of B cells induced by sODN. B cells (25×10^3/well) were cultured with IL-2 in the presence or absence of DSP30 for 8 d, after which RNA was harvested and analyzed for the content of heavy chain variable gene products by RT-PCR using μchain- and V_H-specific primers. The data are representative of three separate experiments, each using B cells from an individual donor.

mRNA for all six major V_H families. These results support the conclusion that the B cell response to sODNs is polyclonal.

Anti-DNA antibodies are induced by sODNs and bind to both calf thymus DNA and sODNs. The next experiments investigated whether sODN-activated B cells produce anti-DNA Ab. B cells were cultured with sODNs with or without IL-2 and analyzed for anti-DNA Ab secretion. B cells cultured with activated T cells were used as a positive control (Fig. 8). B cells cultured alone or with IL-2 or with DSP25 alone did not produce anti-DNA Ab. By contrast, the three most stimulatory sODNs (1082, 2105, and DSP30) induced anti-DNA Ab production, and production of these autoantibodies was enhanced by coculture with IL-2. Anti-DNA Ab production by B cells stimulated by sODNs and IL-2 was even greater than that induced by activated T cells. Since the latter induced more IgM, the sODNs stimulated a greater ratio of IgM anti-DNA to IgM than anti–CD3-activated T cells.

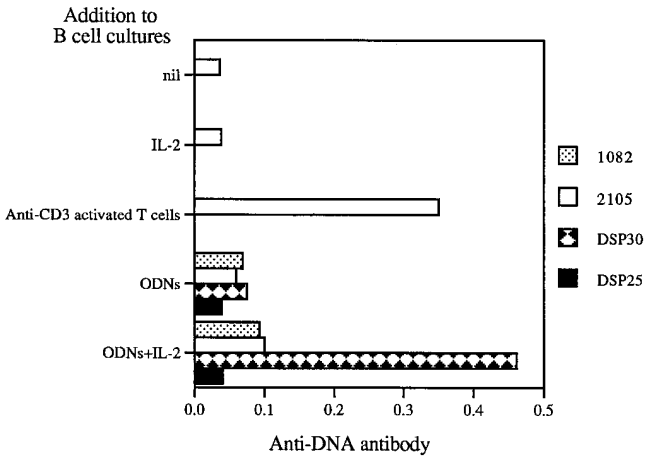


Figure 8. Anti-DNA Ab secretion induced by sODNs. B cells were cultured with sODNs at their optimal concentrations (1 μg/ml for DSP30 and 5 μg/ml for others) in the presence or absence of IL-2 for 14 d. Additional sODNs (1 μg/ml) were added at days 2 and 5 to induce maximal production of anti-DNA antibody. Anti-DNA Ab in the supernatant was analyzed by ELISA using calf thymus DNA as substrate. B cells cultured with activated T cells were used as a positive control. The anti-DNA antibody of the negative control (no supernatant) is 0.049. The results of one of three experiments, each using cells from an individual donor, with similar findings are shown.

To investigate the reactivity of induced anti-DNA Abs with sODNs, supernatants of activated B cells were harvested and assessed for binding to sODNs. As can been seen in Fig. 9, B cells cultured alone, with IL-2, or with DSP25 and IL-2 did not produce anti–ODN Ab. In contrast, the stimulatory sODNs (1082, 2105, and DSP30) induced production of Abs that bound to all three of the stimulatory sODNs as well as the other sODNs, such as DSP25. These results indicate that sODNs activate B cells committed to production of anti-DNA antibodies. However, specificity for the activating sODNs was not apparent.

Proliferation and IgM production induced by sODNs bound to Sepharose beads. To assess whether sODNs induce B cell activation by binding to cell surface receptors or require internalization, the activity of beads containing covalently coupled DSP30 was tested. As shown in Fig. 10, beads coated with DSP30 were as effective as DSP30 at inducing B cell proliferation. In addition, Sepharose-bound DSP30 induced production of similar amounts of IgM as soluble DSP30, while control glycine-coated beads induced neither proliferation nor IgM production. These results indicate that sODNs stimulate B cells by binding to cell surface receptors and suggest that cell entry of sODN is not necessary.

Maximal B cell activation is induced by sODNs containing a TCG element or CpG motif. To identify sODN sequences necessary for B cell responses, we focused on DSP30 and examined the stimulatory activity of various related sequences. We synthesized three 15-bp sODNs (DSP42, DSP43, and DSP44) containing the sequences corresponding to the 5′, central, and 3′ regions of DSP30. DSP42, the 5′ 15 bp of DSP30 stimulated comparable DNA synthesis and IgM production as the full-length 27 mer, whereas DSP43 and DSP44, the central and 3′ 15 mers, were less stimulatory (Table II). Comparison of the

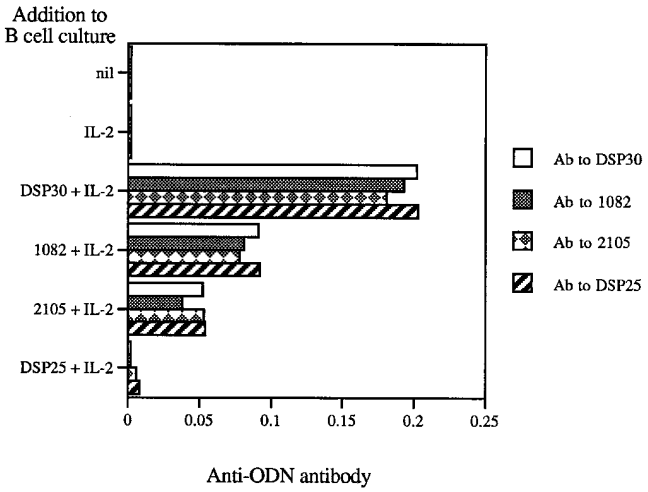


Figure 9. Anti-ODN Ab secretion induced by sODNs. Similar experiments were done as in Fig. 8, except the anti–sODN Ab in the supernatant was analyzed by ELISA. To accomplish this, the sODNs, DSP30 (1 μg/ml), 1082 (5 μg/ml), 2105 (5 μg/ml), and DSP25 (5 μg/ml) that were used to stimulate B cells were used to coat the ELISA plates. The anti-ODN antibody of the negative control (no supernatant) is 0.054, 0.061, 0.064, and 0.065 for DSP30-, 1082-, 2105-, and DSP25-coated plates, respectively. The data shown were derived by subtraction of the anti-ODN antibody of the negative control. The results of one of three experiments, each using cells from a separate donor, with similar findings are shown.

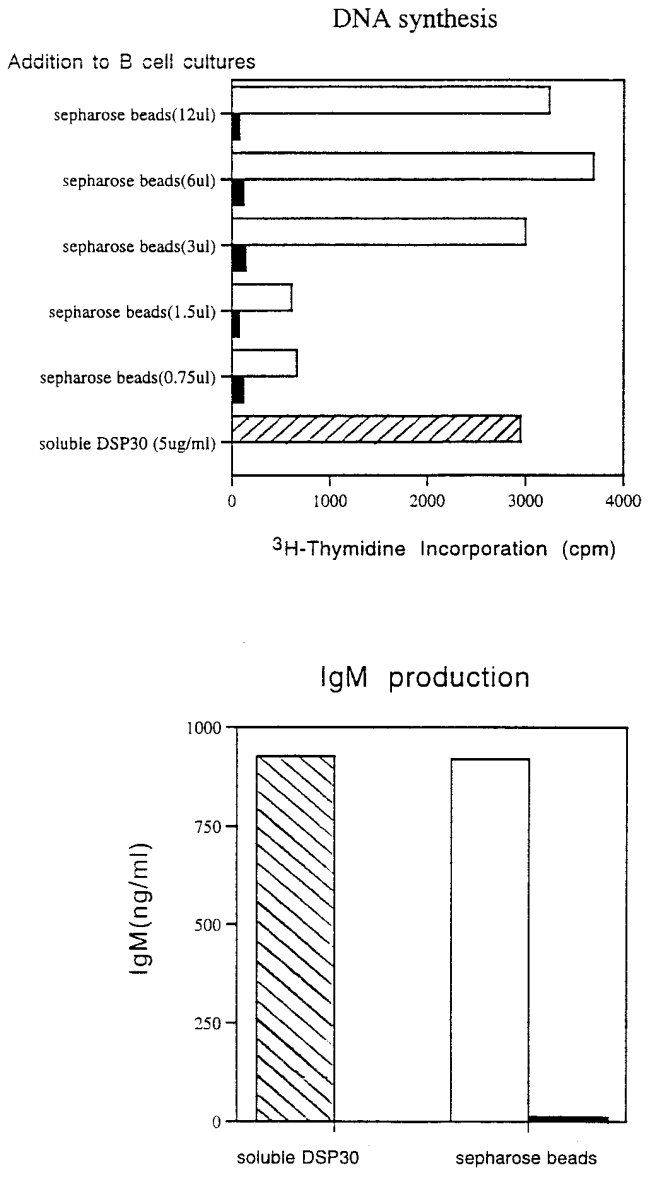


Figure 10. Proliferation and IgM production induced by Sepharose-bound sODNs. B cells were cultured with soluble DSP30 (5 μg/ml) or DSP30-coated Sepharose beads (6 μl, 5 μg/ml) or glycine-coated beads and proliferation and Ig production were assessed. B cells alone incorporated < 100 cpm of [^{3}H]thymidine and produced < 10 ng/ml of Ig. The results of one of three experiments, each using cells from an individual donor, with similar findings are shown. □, DSP30-coated beads; ■, glycine-coated beads.

sequences indicated that TCGTCG was present only in DSP42, but not in the others. To determine whether this motif was stimulatory, the activity of additional 15-bp sODNs was tested. Of these compounds, $(TCG)_5$ induced equal or greater B cell proliferation and Ig production than DSP30, whereas a 5′ TCG doublet followed by nine 3′ Gs failed to stimulate B cell proliferation or Ig production. Additional sODNs, in which the TCG repeat was altered to ACG, TCC, or TGG, had no stimulatory activity (Table II).

To examine the minimal length of active sODNs, additional sODNs that contained two to four repeats of TCG (DSP106–108) were synthesized and tested for their stimulatory activity. As shown in Table III, DSP107 that contains three TCG repeats was stimulatory, but required 10–20-fold the concentration of $(TCG)_4$ or $(TCG)_5$ to achieve comparable stimulation. In contrast, a tandem TCG (DSP106) had minimal stimulatory activity. Taken together, these data indicate that $(TCG)_n$, where $n \geq 3$, is a minimal stimulatory element.

The next experiments investigated whether other motifs might stimulate human B cell activation. Since GACGTT, GACGTC, TGACGTT, or TGACGTC were the most stimulatory motifs for murine B cells (6), additional sODNs containing two or three repeats of these motifs were synthesized and examined for their capacity to induce human B cell proliferation. As shown in Table III, DSP100 $(GACGTT)_3$, DSP101 $(TGACGTT)_3$, DSP102 $(GACGTC)_2$, and DSP103 $(GACGTC)_3$ induced marked B cell proliferation comparable to that induced by DSP30. The shortest sODN of this group (DSP102) required 5–25 times the concentration of the others to achieve maximal stimulation. These data suggest that the motifs that stimulate murine B cell can also stimulate human B cells. These motifs are not unique in this regard, however, since DSP30, which does not contain the entire motif, also induced maximal activation of human B cells. The minimal stimulatory sequence in the mouse $(CG)_n$, however, was not maximally active on human B cells. Thus, as shown in Table III, DSP109 $(CG)_3$, DSP110 $(CG)_4$, and DSP111 (CGCGCGCGCGCGC) induced only minimal proliferation of human B cells. These findings indicate that $(CG)_n$ is not sufficient for maximal sODN-induced B cell activation.

In summary, these results indicate that either $(TCG)_n$ or tandem repeats of the entire CpG motif induced maximal sODN-induced activation of human B cells. However, maximal sODN-induced activation could also be induced without the entire CpG motif, whereas $(CG)_n$ dinucleotides were insufficient to induce maximal sODN-induced B cell activation.

Discussion

The current data demonstrate: (*a*) 12 of 47 phosphorothioate ODNs induce significant polyclonal activation of human B cells; (*b*) these sODNs induce activation of > 95% of human B cells, but not T cells; (*c*) activated B cells express all six of the major Ig V_H gene families; and (*d*) immobilized sODNs stimulate B cells as effectively as the free sODN. These results indicate that sODNs can directly induce polyclonal activation of human B cells by binding to as yet unknown B cell surface receptors.

The mechanism of polyclonal activation of human B cells induced by specific ODNs is not clear. Studies in the mouse showed that ODNs linked to a solid support are not stimulatory (6). In addition, there was no difference between the binding of labeled active ODNs and inactive ODNs (6). These data suggested that ODNs do not bind to surface receptors and that cell uptake is required for stimulation. However, the issue remains controversial, since it was shown that oligo- and polynucleotides cannot diffuse through lipid membranes, although they may enter by bulk pinocytosis (26). In contrast, the current findings demonstrate that sODNs bound to Sepharose beads induce equivalent activation of human B cells as the free sODN, indicating that entry into human B cells is not necessary for the stimulatory effect of sODNs. Because the active sODNs

Table II. Stimulation of B cells by Oligodeoxynucleotides

Cmpd	Sequence of oligodeoxynucleotide	Size	^{3}H-labeled TdR incorporation	IgM secretion
		base pairs	$\times 10^{-3}$ *cpm*	*ng/ml*
DSP30	5′-TCGTCGCTGTCTCCGCTTCTTCTTGCC-3′	27	14.7	5,492
DSP42	5′-TCGTCGCTGTCTCCG-3′	15	12.3	4,633
DSP43	5′-CTGTCTCCGCTTCTT-3′	15	2.6	433
DSP44	5′-CCGCTTCTTCTTGCC-3′	15	4.7	482
DSP49	5′-TCGTCGTCGTCGTCG-3′	15	13.0	5,541
DSP50	5′-TCGTCGGGGGGGGGG-3′	15	0.3	0
DSP54	5′-ACGACGACGACGACG-3′	15	0.2	0
DSP55	5′-TCCTCCTCCTCCTCC-3′	15	2.6	159
DSP56	5′-TGGTGGTGGTGGTGG-3′	15	0.3	0
DSP57	5′-TAGTAGTAGTAGTAG-3′	15	0.4	0

Various 15 mer sODNs were synthesized and examined for their capacity to induce B cell proliferation and Ig production in the presence of IL-2. The results of one of three experiments with similar findings are shown. Cmpd, compound.

form a very stable covalent bond with the beads, it is unlikely that free or released sODNs account for the stimulatory activity. It is more likely that the sODN bound to surface receptors on B cells and, thereby, stimulated B cell activation. It remains possible, however, that entry of sODNs into cells may also provide stimulatory signals and another pathway of activation.

The nature of the putative B cell receptor or receptors involved in sODN-induced B cell activation is currently unknown. Recent evidence indicates that binding of ODNs and DNA to cells is a saturable and specific process dependent on the size of the ODN. This evidence implies the existence of oligonucleotide receptors on the B cell surface (27). In fact, a

Table III. Stimulation of B cells by 13 ODNs

Cmpd	Sequence of ODNs	Size	Concentration inducing maximum response	Mean [^{3}H]thymidine incorporation
		base pairs	*μg/ml*	*cpm*
Marked proliferation				
DSP108	5′-TCG TCG TCG TCG-′3	12	5	7,321
DSP101	5′-TGA CGT TTG ACG TTT GAC GTT-3′	21	1	6,704
DSP107	5′-TCG TCG TCG-3′	9	100	6,613
DSP100	5′-GAC GTT GAC GTT GAC GTT-3′	18	5	5,272
DSP102	5′-GAC GTC GAC GTC-3′	12	25	4,523
DSP103	5′-GAC GTC GAC GTC GAC GTC-3′	18	5	4,137
DSP99	5′-GAC GTT GAC GTT-3′	12	10	3,575
DSP30	5′-TCG TCG CTG TCT CCG CTT CTT CTT GCC-3′	27	1	6,267
DSP49	5′-TCG TCG TCG TCG TCG-3′	15	10	5,112
Moderate proliferation				
DSP105	5′-GGG GGG GGG GGG ACC GGT GGG GGG GGG GGG GGG-3′	30	10	2,636
DSP104	5′-GGG GGG GGG GGG AAC GTT GGG GGG GGG GGG-3′	30	10	2,263
Minimal proliferation				
DSP110	5′-CGC GCG CG-3′	8	100	1,215
DSP109	5′-CGC GCG-3′	6	100	1,051
DSP111	5′-CGC GCG CGC GCG CGC-3′	15	50	1,042
DSP106	5′-TCG TCG-3′	6	75	658
B Alone				244

Various sODNs were synthesized and examined for their capacity to induce B cell proliferation over a wide range of concentrations. The concentrations of each sODN inducing maximal B cell DNA synthesis and B cell proliferation induced by each sODN at its optimal concentration are shown. Data shown are the mean [^{3}H]thymidine incorporation of three or more experiments. The categories were defined as follows: marked $>$ 3,010 cpm, 1,510 cpm $<$ moderate $<$ 3,000 cpm, minimal $<$ 1,500 cpm. There was no significant difference in the magnitude of [^{3}H]thymidine incorporation by any pair of sODNs within each category, although there were significant differences between proliferation induced by at least one sODN from different categories ($P < 0.05$) and between [^{3}H]thymidine incorporation induced by any sODN and that manifested by B cells alone. Cmpd, compound.

75–80-kD nucleic acid binding protein was found on the monocytic leukemia cell line U937 and the T cell acute lymphoblastic leukemia cell line, CEM. A DNA binding protein was also isolated by chromatography on oligo(dT) cellulose from HL60 cells and found to bind polynucleotides, oligonucleotides, as well as phosphorothioate derivatives of oligonucleotides and mononucleotides; this protein, however, did not bind nucleosides, deoxyriboses, or methylphosphonate oligonucleotides (27–30).

In other studies, a 28–30-kD nucleic acid membrane binding protein was detected by chromatography and on immunoblots of cell membrane preparations of peripheral blood cells including neutrophils, adherent mononuclear cells, E rosetting cells, and non-E rosetting cells (30–32). In addition, Beltinger et al. (33), using immunochemical and ultrastructural techniques, found that K562 cells express five cell surface proteins, ranging in size from 20 to 143 kD, that bound to phosphorothioate derivatives of an antisense to c-myb. These results all point to the presence of surface proteins that bind to DNA and ODNs and are consistent with the conclusion that binding one or more of these receptors may lead to polyclonal B cell activation. Preliminary evidence suggests that Sepharose-bound sODNs precipitate a series of four distinct bands from the surface of iodinated B cell lines and is consistent with the conclusion that the sODNs can bind to as yet unidentified B cell surface receptors. The likelihood that the various sODNs may stimulate B cells by binding to the same receptor or set of receptors is supported by the observation that combinations of maximally stimulatory sODNs (DSP101 and DSP108, DSP30 and 1082, and DSP30 and DSP49) had no additive or synergistic effect on human B cell responses (data not shown).

The current data also suggest that human B cell activation induced by sODNs is different than that observed in the mouse. First, the basic activation motif appears to be different in man and mouse. It has been reported in mice that *E. coli* genomic DNA and ODNs containing an unmethylated CpG motif induce B cells to proliferate and secrete immunoglobulins both in vivo and in vitro. The motif that induces murine B cell activation contains an unmethylated CpG dinucleotide flanked by two 5′ purines (preferably a GpA dinucleotide) and two 3′ pyrimidines (preferably a TpC or TpT dinucleotide) (6). In the current study, bacterial DNA failed to induce B cell activation. Furthermore, although the entire CpG motif induced maximal human B cell activation, sODNs lacking this motif were also active. These results suggest that the CpG motif that induces murine B cell activation can induce, but is not necessary for, human B cell activation. In addition, $(CpG)_n$, which is a potent stimulator of murine B cells, does not appear to be capable of inducing maximal human B cell activation. Moreover, in preliminary experiments, sODNs that lacked CG dinucleotides (5′-ACC ACA CTG GTA TTT CAC AC-3′ and 5′-GAG ACC CTG AAC AGT TGA TC-3′) induced human B cell proliferation, suggesting that CG dinucleotides may not be necessary for human B cell activation. It should be noted, however, that these sODNs did not induce maximal DNA synthesis (2,792 and 2,674 cpm, respectively), suggesting the possibility that CG dinucleotides may be necessary for maximal sODN-induced human B cell proliferation.

The mechanism of B cell activation also appears to be different between man and mouse. Although binding to surface receptors does not seem to be involved in the mouse (6), immobilized sODNs can induce maximal human B cell activation. These findings indicate that in man, sODNs stimulate B cell activation by binding to surface receptors, although this issue remains unresolved in mouse.

There are various hypotheses concerning the mechanism of human B cell activation induced by specific sODNs. One is that the stimulatory activity of sODNs results from a specific nucleotide sequence (i.e., the primary structure of sODNs). The other is that sODNs induce human B cell activation by forming certain secondary or tertiary structures. The current data show that $(TCG)_n$ or the entire CpG motif induced maximal activation of human B cells and this maximal activation could not be induced by any phosphorothioate that lacked one or the other of these sequences. Of note, the stimulatory sODNs 1082 and 2105 both contain portions of these motifs, although additional stimulatory motifs may account for their activity. These results suggest that human B cell activation is not induced by the negatively charged phosphorothioate backbone. Rather, it appears that specific nucleotide sequences are involved in the activation. Additional evidence is consistent with this conclusion. Thus, for example, DSP17, the diester form of 1082, did not induce maximal activation of human B cells, but it was active to a modest degree. Since the half-life of diesters is estimated to be only 4 h in culture vs 14 h for phosphorothioates (34–36), DSP17 may be degraded before the activation signals are totally delivered. These results are consistent with the conclusion that human B cell activation is related to the specific sequences of ODNs, and not merely the phosphorothioate backbone. It remains possible, however, that the phosphorothioate backbone provides a permissive influence that permits sequence-specific polyclonal human B cell activation.

It is also possible that secondary or tertiary structures rather than a specific sequence may be involved in stimulating human B cells. To examine this possibility, the sequences of the 47 ODNs were analyzed to determine whether they could potentially form hairpins or other secondary structures. The results showed that 1082, DSP100–103, and DSP111 are candidates to form hairpins, whereas the other ODNs are likely to remain in a linear form. DSP30, 2105, DSP49, and DSP42 are not predicted to form stable hairpins, but may form unstable hairpins. This observation implies that formation of hairpins may contribute to the B cell stimulatory activity of sODNs. Since four of the most stimulatory sODNs are not predicted to form stable hairpins, however, it is likely that primary sequence also plays a role. Clearly, the capacity to form a stable hairpin cannot be the only determinant of stimulatory capacity, as DSP111, which is predicted to form a stable hairpin, is only minimally stimulatory.

Besides the delineation of the stimulatory capacity of certain sODNs, the current findings may be important for understanding the pathogenesis of SLE. Although this disease is characterized by high avidity anti-DNA antibody (12, 13), the respective roles of a putative DNA antigen–driven response and polyclonal activation remain uncertain. The current data show that certain sODNs have the ability to induce polyclonal activation of human B cells. These activated B cells can produce anti-DNA Abs and anti–sODN Abs, although those antibodies appear to be very low avidity (data not shown). These results imply that certain ODNs or equivalent molecules may play a role in the early stage of SLE by inducing the production of low avidity anti-DNA antibodies that may provide a substrate for subsequent T cell–directed somatic mutation with

the resultant production of high avidity pathogenic anti-DNA antibodies.

This finding may also be relevent to the medicinal use of nucleic acid both in antisense strategies and DNA vaccination. In recent years, antisense ODNs have attracted attention as highly specific agents to block expression of specific genes. However, recent evidence suggests that antisense ODNs can have nonspecific effects, either inhibiting or stimulating cellular function (9–11, 37). For instance, an antisense to a portion of the rev region of the HIV genome (i.e., DSP30, the most active sODN in the current study) induced activation of murine B cells in vitro. After intravenous injection of this compound, mice developed massive splenomegaly and polyclonal hypergammaglobulinemia within 2 d (9). Moreover, simultaneous addition of certain sODNs with Ag enhanced Ag-specific murine B cell activation, suggesting that sODNs could provide costimulatory signals for Ag-specific murine B cell activation (6). To date, the nonspecific stimulatory effect of antisense sODNs has not been examined in detail with human B cells. The findings that human B cell activation is induced by sODNs containing $(TCG)_n$, the full CpG motif, and perhaps other motifs provide important information in developing specific antisense ODNs that lack nonspecific stimulatory activity. On the other hand, the inclusion of stimulatory motifs may be important in the development of DNA-based vaccines.

Acknowledgments

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References

1. Stollar, B.D. 1986. Antibodies to DNA. *CRC Crit. Rev. Biochem.* 20:1–36.

2. Stollar, B.D. 1975. The specificity and applications of antibodies to helical nucleic acids. *CRC Crit. Rev. Biochem.* 3:45–69.

3. Rich, A., A. Nordheim, and A.H.-J. Wang. 1984. The chemistry and biology of left-handed Z-DNA. *Annu. Rev. Biochem.* 53:791–846.

4. Zarling, D.A., D.J. Arndt-Jovin, M. Robert-Nicoud, L.P. McIntosh, R. Thomae, and T.M. Jovin. 1984. Immunoglobulin recognition of synthetic and natural left-handed Z DNA conformations and sequences. *J. Mol. Biol.* 176:369–415.

5. Frappier, L., G.B. Price, R.G. Martin, and M. Zannis-Hadjopoulos. 1989. Characterization of the binding specificity of two anticruciform DNA monoclonal antibodies. *J. Biol. Chem.* 264:334–341.

6. Krieg, A.M., A. Yi, S. Matson, T.J. Waldschmidt, G.A. Bishop, R. Teasdale, G.A. Koretzky, and D.M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B cell activation. *Nature (Lond.).* 371:546–549.

7. Messina, J.P., and D.S. Pisetsky. 1991. Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA. *J. Immunol.* 147:1759–1764.

8. Messina, J.P., G.S. Gilkeson, and D.S. Pisetsky. 1993. The influence of DNA structure on the in vitro stimulation of murine lymphocytes by natural and synthetic polynucleotide antigens. *Cell. Immunol.* 147:148–157.

9. Branda, R.F., A.L. Moore, L. Mathews, J.J. McCormarck, and G. Zon. 1993. Immune stimulation by an antisense oligomer complementary to the rev gene of HIV-1. *Biochem. Pharmac.* 45:2037–2043.

10. Pisetsky, D.S., and C.F. Reich. 1993. Stimulation of murine lymphocyte proliferation by a phosphorothioate oligonucleotide with antisense activity for herpes simplex virus. *Life Sci.* 54:101–107.

11. Krieg, A., W.C. Gause, M.F. Gourley, and A.D. Steinberg. 1989. A role for endogenous retroviral sequences in the regulation of lymphocyte activation. *J. Immunol.* 143:2448–2451.

12. Tan, E.M. 1982. Autoantibodies to nuclear antigens (ANA): their immunobiology and medicine. *Adv. Immunol.* 33:167–240.

13. Koffler, D., P.H. Schur, and H.G. Kunkel. 1967. Immunological studies concerning the nephritis of systemic lupus erythematosus. *J. Exp. Med.* 126:607–624.

14. Krieg, A.M. 1995. CpG DNA: a pathogenic factor in systemic lupus erythematosus. *J. Clin. Immunol.* 15:284–292.

15. Fournie, G.J., J.-M. Dueymes, M. Gayral-Taminh, J.P. Pourrat, M. Mignon-Conte, and J.J. Conte. 1984. Anti-DNA antibody idiotypes in SLE. *Lancet.* 2:821–822.

16. McCoubrey-Hoyer, A., T.B. Okarma, and H.R. Holman. 1984. Partial purification and characterization of plasma DNA and its relation to disease activity in systemic lupus erythematosus. *Am. J. Med.* 77:23–33.

17. Thuong, N.T., and U. Asseline. 1985. Chemical synthesis of natural and modified oligodeoxynucleotides. *Biochemie.* 67:673–684.

18. Rosenberg, S.A., and P.E. Lipsky. 1979. Monocyte dependence of pokeweed mitogen-induced differentiation of immunoglobulin-secreting cells from human peripheral blood mononuclear cells. *J. Immunol.* 122:926–931.

19. Thiele, D.L., M. Kurosaka, and P.E. Lipsky. 1983. Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: delineation by its sensitivity to the lysosomotrophic agent, L-leucine methyl ester. *J. Immunol.* 131:2282–2290.

20. Thiele, D.L., and P.E. Lipsky. 1985. Regulation of cellular function by products of lysosomal enzyme activity: elimination of human natural killer cells by a dipeptide methyl ester generated from L-leucine methyl ester by monocytres or polymorphonuclear leukocytes. *Proc. Natl. Acad. Sci. USA.* 82:2468–2472.

21. Galili, U., and M. Schlesinger. 1974. The formation of stable E rosettes after neuraminidase treatment of either human peripheral blood lymphocytes or of sheep red blood cells. *J. Immunol.* 112:1628–1634.

22. Amoroso, K., and P.E. Lipsky. 1990. Frequence of human B cells that differentiate in response to anti-CD3-activated T cells. *J. Immunol.* 145:3155–3161.

23. Engvall, E., and P. Perlman. 1971. Enzyme-linked immunosorbent assay (ELISA): quantitative assay of immunoglobulin G. *Immunochemistry.* 8:871–874.

24. Tohma, S., and P.E. Lipsky. 1991. Analysis of the mechanisms of T cell–dependent polyclonal activation of human B cells: induction of human B cell responses by fixed activated T cells. *J. Immunol.* 146:2544–2552.

25. Kung, P., G. Goldstein, E.L. Reinherz, and S.F. Schlossman. 1979. Monoclonal antibodies defining distinctive human T cell surface antigens. *Science (Wash. DC).* 206:347–349.

26. Akhtar, S., S. Basu, E. Wickstrom, and R.L. Juliano. 1991. Interaction of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes). *Nucleic. Acids Res.* 19:5551–5559.

27. Loke, S.L., C.A. Stein, X.H. Zhang, K. Mori, M. Nakanishi, C. Subasinghe, J.S. Cohen, and L.M. Neckers. 1989. Characterization of oligonucleotide transport into living cells. *Proc. Natl. Acad. Sci. USA.* 86:3474–3478.

28. Yakubov, L.A., E.A. Deeva, V.F. Zarytova, E.M. Ivanova, A.S. Ryte, L.V. Yurchenko, and V.V. Vlassov. 1989. Mechanism of oligonucleotide uptake by cells: involvement of specific receptors? *Proc. Natl. Acad. Sci. USA.* 86:6454–6458.

29. Geselowitz, D.A., and L.M. Neckers. 1992. Analysis of oligonucleotide binding, internalization, and intracellular trafficking utilizing a novel radiolabeled crosslinker. *Antisense Res. Dev.* 2:17–25.

30. Gasparro, F.P., R. Dall'amico, M. O'Malley, P.W. Heald, and R.L. Edelson. 1990. Cell membrane DNA: a new target for psoralen photoadduct formation. *Photochem. Photobiol.* 52:315–321.

31. Bennett, R.M., G.T. Gabor, and M.M. Merritt. 1985. DNA binding to human leukocytes: evidence for a receptor-mediated association, internalization, and degradation of DNA. *J. Clin. Invest.* 76:2182–2190.

32. Bennet, R.M., S.H. Hefeneider, A. Bakke, M. Merritt, C.A. Smith, D. Mourich, and M.C. Heinrich. 1988. The production and characterization of murine monoclonal anticodies to a DNA receptor on human leukocytes. *J. Immunol.* 140:2937–2942.

33. Beltinger, C., H.U. Saragovi, R.M. Smith, L. LeSauteur, N. Shah, L. DeDionisio, L. Christensen, A. Raible, L. Jarett, and A.M. Gewirtz. 1995. Binding, uptake, and intracellular trafficking of phosphorothioate-modified oligodeoxynucleotides. *J. Clin. Invest.* 95:1814–1823.

34. Zhao, Q., S. Matson, C.J. Herrera, E. Fisher, H. Yu, and A.M. Krieg. 1993. Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides. *Antisense Res. Dev.* 3:53–66.

35. Campbell, J.M., T.A. Bacon, and E. Wickstrom. 1990. Oligodeoxynucleotide phosphorothioate stability in subcellular extracts, culture media, sera and cerebrospinal fluid. *J. Biochem. Biophys. Methods.* 20:259–267.

36. Agrawal, S., J. Temsamani, and J.Y. Tang. 1991. Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice. *Proc. Natl. Acad. Sci. USA.* 88:7595–7599.

37. Storey, A., D. Oates, L. Banks, L. Crawford, and T. Crook. 1991. Antisense phosphorothioate oligonucleotides have both specific and non-specific effects on cells containing human papillomavirus type 16. *Nucleic Acids Res.* 19:4109–4114.

38. Yamamoto, S., T. Yamamoto, T. Kataoka, E. Kuramoto, O. Yana, and T. Tokunaga. 1992. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J. Immunol.* 148:4072–4076.

39. Kimura, Y., K. Sonehara, E. Kuramoto, T. Makino, S. Yamamoto, T. Yamamoto, T. Kataoka, and T. Tokunaga. 1994. Binding of oligoguanylate to scavenger receptors is required for oligonucleotides to augment NK cell activity and induce IFN. *J. Biochem. (Tokyo).* 116:991–994.