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# Effect of Suppressive DNA on CpG-Induced Immune Activation[1]

**Hiroshi Yamada, Ihsan Gursel, Fumihiko Takeshita, Jackie Conover, Ken J. Ishii, Mayda Gursel, Saoko Takeshita, and Dennis M. Klinman[2]**

**Bacterial DNA and synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs stimulate a strong innate immune response. This stimulation can be abrogated by either removing the CpG DNA or adding inhibitory/suppressive motifs. Suppression is dominant over stimulation and is specific for CpG-induced immune responses (having no effect on LPS- or Con A-induced activation). Individual cells noncompetitively internalize both stimulatory and suppressive ODN. Studies using ODN composed of both stimulatory and suppressive motifs indicate that sequence recognition proceeds in a 5′→3′ direction, and that a 5′ motif can block recognition of immediately 3′ sequences. These findings contribute to our understanding of the immunomodulatory activity of DNA-based products and the rules that govern immune recognition of stimulatory and suppressive motifs.** ***The Journal of Immunology*****, 2002, 169: 5590–5594.**

Bacterial DNA contains bioactive CpG motifs that interact with Toll-like receptor 9 to trigger an innate immune response (1–6). While CpG-induced immunity helps protect the host from pathogenic infections (7–10), exposure to stimulatory motifs can have deleterious consequences, ranging from autoimmune disease to death (11–15).

Krieg et al. (16) were the first to report that neutralizing or suppressive motifs can selectively block CpG-mediated immune stimulation. These motifs inhibited cytokine production in vitro and reduced the adjuvant effects of CpG DNA in vivo. Suppressive motifs are rich in polyG or -GC sequences, tend to be methylated, and are present in the DNA of mammals and certain viruses (16–18).

Little is known about the kinetics, magnitude, or nature of the immune inhibition elicited by suppressive motifs. Current studies establish that the immunostimulatory activity of CpG DNA can be reversed within several hours by removal of stimulatory DNA or addition of suppressive DNA. Stimulatory and suppressive DNA binds to and interacts with the same cells. When both sequence types are present on a single strand of DNA, recognition proceeds in a 5′→3′ direction. Suppression is generally dominant over stimulation, although a motif in the 5′ position can interfere with recognition of a motif immediately downstream. Understanding the rules governing cellular responses to stimulatory and suppressive motifs should facilitate the design of oligodeoxynucleotides (ODN)[3] for therapeutic uses.

## Materials and Methods

### *Animals*

Female BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were housed under specific pathogen-free conditions and were used at 8–20 wk of age. All studies involved protocols approved by the Center for Biologics Evaluation and Research animal care and use committee.

### *Oligodeoxynucleotides*

Studies used phosphorothioate-modified ODNs that were synthesized at the Center for Biologics Evaluation and Research core facility (19). The following ODNs were used: immunostimulatory, $ODN_{1466}$ (TCAACGTTGA) and $ODN_{1555}$ (GCTAGACGTTAGCGT); control, $ODN_{1471}$ (TCAAGCTTGA) and $ODN_{1612}$ (GCTAGAGCTTAGGCT); and suppressive, $ODN_{1502}$ (GAGCAAGCTGGACCTTCCAT) and $ODN_{H154}$ (CCTCAAGCTTGAGGGG). The underlined bases represent the 10-mer sequences that were incorporated into complex multideterminant ODN used in some experiments. There was no detectable protein or endotoxin contamination of these ODN.

Mammalian DNA was purified from BALB/c spleens (Wizard Genomic DNA purification kit; Promega, Madison, WI). *Escherichia coli* DNA was obtained from Life Technologies (Gaithersburg, MD). Endotoxin contamination in these preparations was <0.1 U/ml after purification (20). Double-stranded DNA was converted to ssDNA by heat denaturing at 95°C for 5 min, followed by immediate cooling on ice.

### *Cytokine ELISAs*

Spleen single-cell suspensions were washed three times and resuspended in RPMI 1640 supplemented with 5% heat-inactivated FCS, 1.5 mM L-glutamine, and 100 U/ml of penicillin/streptomycin. Cells ($5 \times 10^5$/well) were cultured in flat-bottom microtiter plates (Costar, Corning, NY) with 1 $\mu$M ODN for 18–24 h. Culture supernatants were collected, and cytokine levels were measured by ELISA. In brief, 96-well Immulon H2B plates (Thermo LabSystems, Franklin, MA) were coated with cytokine-specific Abs and blocked with PBS 1% BSA as previously described (21). Culture supernatants were added, and bound cytokine was detected by the addition of biotin-labeled secondary Abs, followed by phosphatase-conjugated avidin and a phosphatase-specific colorimetric substrate (PNPP; Pierce, Rockford, IL). Standard curves were generated using recombinant cytokines. The detection limit for these assays was 0.8 U/ml for IFN-$\gamma$, 0.1 ng/ml for IL-6, and 0.1 ng/ml for IL-12. All assays were performed in triplicate.

### *Cytokine-specific ELISPOT assays*

A spleen single-cell suspension prepared in RPMI 1640 plus 5% FCS was serially diluted onto plates precoated with anti-cytokine Abs (21). Cells were incubated with 1 $\mu$M ODN at 37°C for 8–12 h, and the secretion of cytokine was detected colorimetrically as previously described (21).

### *Cell surface binding and internalization of ODN*

Spleen cells ($2 \times 10^6$/ml) were incubated with 1 $\mu$M of unlabeled and/or fluorescent-labeled ODN for 10 min at 4°C (binding experiments) or for 1 h at 37°C (uptake experiments). Cells were washed, fixed, and analyzed by FACScan (BD Biosciences, San Jose, CA) (22).

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[3] Abbreviations used in this paper: ODN, oligodeoxynucleotide.



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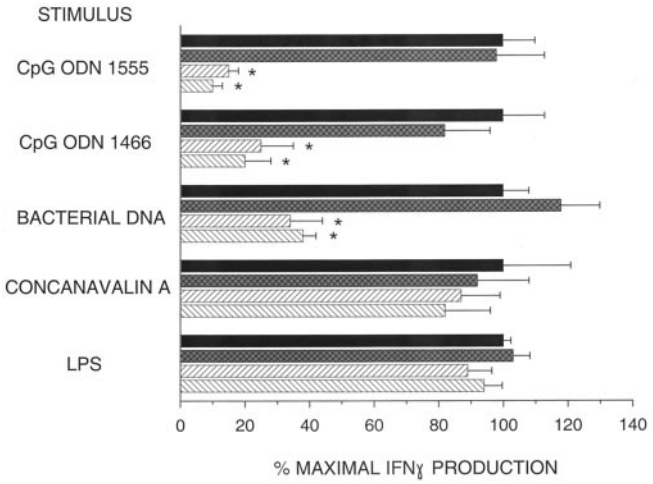


**FIGURE 1.** Effect of suppressive ODN on CpG DNA and mitogen-induced IFN-γ production. BALB/c spleen cells were stimulated with 1 μM CpG ODN ($ODN_{1555}$, $ODN_{1466}$), 50 μg/ml of bacterial DNA, 5 μg/ml of Con A, or 5 μg/ml of LPS. The response of these cultures (■) was compared with that of cells costimulated with 1 μM control $ODN_{1612}$ (▩), suppressive $ODN_{1502}$ (▨), or suppressive $ODN_{H154}$ (▧). The number of IFN-γ-secreting cells was determined by ELISPOT after 18 h. Data represent the average ± SD of triplicate cultures. The experiment was repeated three times with similar results.

### *Statistical analysis*

Statistically significant differences between two groups were determined using the Wilcoxon rank-sum test. When comparing more than two groups, differences were determined using a two-tailed nonparametric ANOVA with Dunn's post-test analysis. A value of $p < 0.05$ was considered significant.

## Results

### *Mammalian DNA suppresses CpG DNA-induced immune activation*

Single-stranded bacterial DNA and synthetic ODN containing unmethylated CpG motifs stimulate immune cells to mature, proliferate, and produce cytokines, chemokines, and Ig (2–5). These effects can be blocked by polyG- and/or GC-rich DNA motifs (16, 23). Scores of ODNs were synthesized and tested to identify motifs that selectively inhibited CpG-induced immune responses. The two most active of these suppressive ODN ($ODN_{1502}$ (GAG CAAGCTGGACCTTCCAT) and $ODN_{H154}$ (CCTCAAGCTT GAGGGG)) were selected for detailed study. As shown in Fig. 1, these suppressive ODN blocked a majority of the IFN-γ production induced by bacterial DNA or CpG ODN ($p < 0.01$). Suppressive ODN were neither toxic nor broadly immunosuppressive, as they did not interfere with the mitogenic activity of LPS or Con A (Fig. 1 and data not shown).

The activity of suppressive ODNs was concentration dependent, with 50% suppression being achieved at a suppressive ODN:CpG ODN ratio of ~1:3 (Fig. 2). To examine the kinetics of this inhibition, suppressive ODN were added to BALB/c spleen cells at various times after CpG-induced stimulation. Maximal inhibition was observed when suppressive ODN were coadministered with CpG ODN, although statistically significant inhibition persisted when suppressive ODN were added up to 3 h later (Fig. 3). These findings suggest that CpG-induced immune activation is an ongoing process and can be inhibited after the stimulatory signal is delivered.

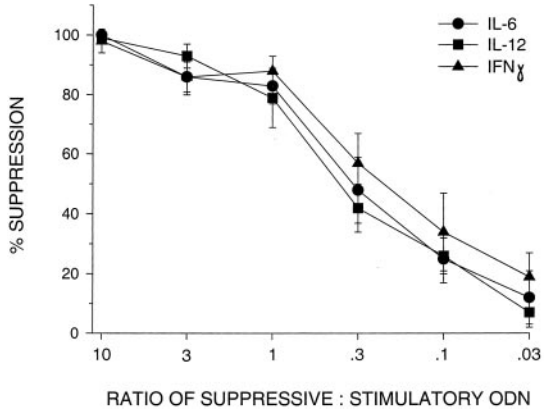


**FIGURE 2.** Concentration effects of suppressive ODN. BALB/c spleen cells were stimulated with 1 μM CpG $ODN_{1555}$ or $ODN_{1466}$ plus increasing amounts of suppressive $ODN_{1502}$ or $ODN_{H154}$. Cytokine levels in culture supernatants were measured by ELISA after 24 h. Results represent the mean ± SD of four different experiments.

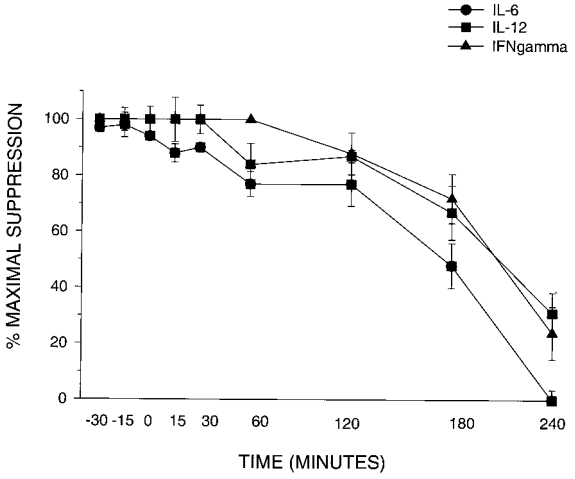


**FIGURE 3.** Kinetics of suppressive ODN. BALB/c spleen cells were stimulated with 1 μM CpG $ODN_{1555}$. At various times, 1 μM suppressive $ODN_{1502}$ was added. Cytokine levels in supernatants were measured by ELISA after 24 h. Results represent the mean of two independent experiments.

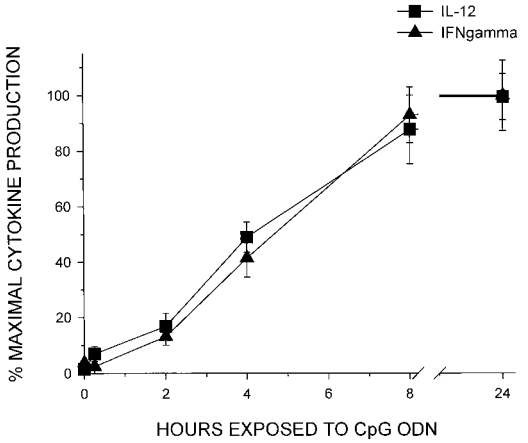


**FIGURE 4.** Effect of removing CpG ODN from cultured cells. CpG $ODN_{1555}$ (1 μM) was added to BALB/c spleen cells at time zero. The cells were washed free of this ODN after various incubation periods. IFN-γ and IL-12 levels in culture supernatants were measured by ELISA after 24 h. Results represent the average ± SD of duplicate cultures. Similar results were obtained in studies of CpG $ODN_{1466}$.

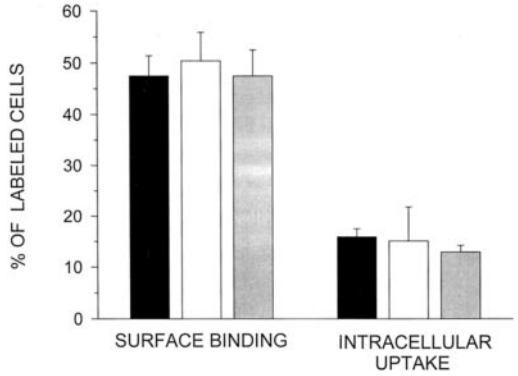


**FIGURE 5.** Suppressive ODN do not block the binding or uptake of CpG ODN. BALB/c spleen cells were incubated with 1 $\mu$M CpG $ODN_{1555}$ (■) plus 1 $\mu$M suppressive $ODN_{1502}$ (▨) or control $ODN_{1612}$ (□) for 2 h. The percentage of cells that bound or internalized the CpG ODN was determined by FACS. Similar results were obtained using CpG $ODN_{1466}$, suppressive $ODN_{H154}$, and control $ODN_{1471}$.

To test this conclusion, spleen cells were incubated with CpG ODN for various periods, and cytokine production was analyzed after 24 h. Cells stimulated with CpG DNA for 8 h produced 90% as much cytokine as cells stimulated continuously for 24 h (Fig. 4). Cells treated with CpG ODN for only 4 h produced half as much cytokine, while cells treated with CpG DNA for ≤2 h showed only minimal activation (Fig. 4). These findings support the conclusion that CpG-induced cellular activation is reversible for several hours.

### *Suppressive ODN do not block CpG ODN uptake or induce the production of inhibitory factors*

The results described above indicate that CpG-induced immune activation can be reversed either by adding suppressive ODN or by removing stimulatory ODN. This suggests that suppressive ODN might block the ongoing uptake of CpG DNA. Yet FACS analysis demonstrated that neither cell surface binding nor internalization of FITC-labeled CpG ODN was significantly reduced by suppressive ODN at concentrations that blocked cytokine production by ~75% (Fig. 5 and data not shown). Moreover, precisely the same cells that bound and internalized CpG ODN interacted with suppressive ODN (Fig. 6).

The possibility that suppressive motifs might induce the production of a factor that blocked CpG-dependent immune stimulation was then investigated. Initial studies established that BALB/c spleen cells preincubated with suppressive ODN remained unresponsive to CpG-induced stimulation for several hours (Table I, line 3). If this nonresponsive state was mediated by a soluble factor (or inhibitory cell-cell interactions) then cells pretreated with suppressive ODN should block CpG-induced stimulation of naive splenocytes. As shown in Table I, cells treated with suppressive ODN had no significant effect on CpG-dependent cytokine production by fresh spleen cells.

Table I. *Effect of mixing cells treated with suppressive vs stimulatory ODN*[a]

| Suppressive ODN | | ODN Added During Culture | % Maximal Cytokine Production | |
|---|---|---|---|---|
| Pretreatment | Fresh Cells | | IL-6 | IL-12 |
| − | + | CpG | 100 ± 13 | 100 ± 6 |
| − | + | Control | 3 ± 2 | 7 ± 2 |
| + | − | CpG | 9 ± 6 | 6 ± 2 |
| + | − | Control | 0 ± 0 | 0 ± 0 |
| + | + | CpG | 86 ± 16 | 105 ± 12 |
| + | + | Control | 0 ± 0 | 0 ± 0 |

[a] BALB/c spleen cells were treated with 1 $\mu$M suppressive $ODN_{H154}$ for 2 h and then washed (first column). These cells were added to naive splenocytes (second column) plus 1 $\mu$M control ($ODN_{1471}$) or CpG ($ODN_{1555}$) ODN. IL-6 and IL-12 levels in culture supernatants were measured by ELISA after 18 h. The percentage of maximal cytokine production was calculated by the formula: (cytokine produced by treatment group) − (background)/(cytokine produced by fresh cells stimulated with CpG ODN) − (background) × 100%, where the background was cytokine levels in fresh cells cultured in medium alone. Results represent the average ± SD of triplicate assays, each standardized to the response induced by bacterial DNA (62 pg/ml IL-6; 134 pg/ml IL-12).

### *Cellular recognition of suppressive vs stimulatory motifs*

The above studies establish that suppressive motifs on one strand of DNA block the immune activation induced by stimulatory motifs on a different strand (i.e., *trans*-suppression). To better understand the interaction between suppressive and stimulatory motifs, ODNs containing both were synthesized. A set of four 20-mer ODNs was constructed in which one of two different CpG motifs was placed immediately 5′ to either of two suppressive motifs (referred to as [CpG-Sup] ODN).

All four of these [CpG-Sup] ODN were stimulatory, triggering murine spleen cells to produce IL-6, IL-12, and IFN-$\gamma$ to the same extent as an ODN of the same length in which the suppressive motif was replaced by a control sequence (i.e., one that was neither stimulatory nor suppressive; Table II). [CpG-Sup] ODNs did not inhibit the immune activation induced by an independent CpG ODN (Table II). These results suggest that a suppressive motif is inactive when located immediately 3′ to a CpG motif on the same strand of DNA.

To better understand this phenomenon, longer ODNs were synthesized in which the CpG and suppressive motifs were separated by progressively longer CT spacers. Adding a 5-base spacer generated an ODN that was still stimulatory (Table III). However, separating the motifs by ≥10 bases yielded ODNs that were suppressive, demonstrated by their ability to block the stimulatory activity of coadministered CpG ODNs (Table III). The trivial possibility that the CT spacer somehow reduced CpG activity was eliminated by substituting a control motif for the 3′ suppressive motif. The resulting ODNs were fully stimulatory (Table III and data not shown).

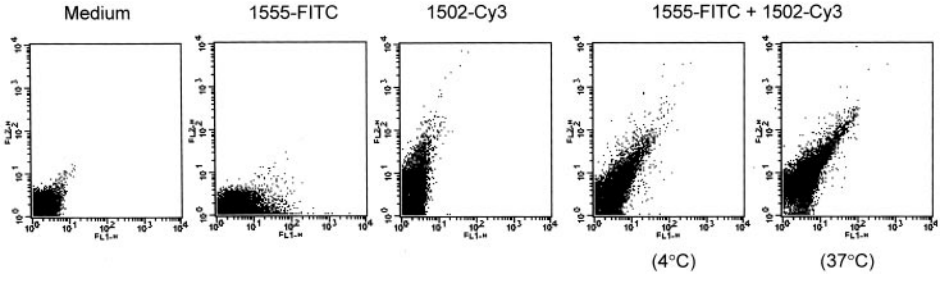


**FIGURE 6.** Binding and internalization of suppressive and CpG ODN. BALB/c spleen cells were incubated with 1 $\mu$M CpG $ODN_{1555}$ and/or 1 $\mu$M suppressive $ODN_{1502}$ at 4°C for 10 min or at 37°C for 2 h. Note that the same cells bound and internalized both CpG and suppressive ODN. Binding increased as the time of incubation was prolonged (Fig. 5).

Table II. *Effect of motif position on immunostimulatory activity*[a]

| Location of Motifs (5′→3′) | No. of Cytokine-Secreting Cells | | |
|---|---|---|---|
| | IL-6 | IL-12 | IFN-γ |
| CpG ODN[b] | 79 ± 3 | 1980 ± 230 | 260 ± 40 |
| [CpG-Sup] ODN[b] | 72 ± 14 | 2080 ± 480 | 230 ± 60 |
| [Sup-CpG] ODN | 0 ± 0 | 140 ± 30 | 0 ± 0 |
| [CpG-Cont] ODN[b] | 64 ± 12 | 2210 ± 130 | 284 ± 34 |
| [Cont-CpG] ODN[b] | 80 ± 11 | 1942 ± 88 | 238 ± 28 |
| [Cont-Sup] ODN | 8 ± 2 | 184 ± 34 | 36 ± 8 |
| [CpG-Sup] ODN + Sup ODN | 4 ± 2 | 226 ± 38 | 28 ± 6 |
| [Sup-CpG] ODN + CpG ODN | 7 ± 3 | 250 ± 32 | 34 ± 9 |

[a] BALB/c spleen cells ($10^6$) were coincubated with 1 μM of each ODN. Complex ODN (20 bp in length) were constructed from 10-mer encoding suppressive (Sup; GAGCAAGCTG and AGCTTGAGGG), stimulatory (CpG; TCACGTTGA and TA GACGTTAG), or control (Cont; TCAAGCTTGA and TAGAGCTTAG) motifs. The number of cytokine-secreting cells per $10^6$ cells was determined by ELISPOT after 24 h of stimulation. Results represent the average ± SD of triplicate assays involving at least two ODN of each type.

[b] Stimulatory ODN, $p < 0.05$.

The impact of placing a suppressive motif 5′ to a CpG motif was then examined. ODNs with a suppressive motif in the 5′ position induced little or no immune activation even when the CpG motif was shifted up to 20 bp downstream from the suppressive motif (Tables II and III). This lack of activity could not be attributed to the 3′ location of the CpG motif, since CpG ODNs with a control sequence at the 5′ end were stimulatory. All ODNs containing a suppressive motif in the 5′ position also inhibited the stimulatory activity of a coadministered CpG ODN (Tables II and III). These findings suggest that the relative positions of stimulatory and suppressive motifs determine the immunomodulatory properties of DNA.

## Discussion

DNA has multiple and complex effects on the immune system. The innate immune response triggered by unmethylated CpG motifs present in bacterial DNA improve host resistance to infectious pathogens (7, 9, 10, 24). Yet CpG stimulation can increase the host's susceptibility to autoimmune disease and death (11–14, 25, 26). This work examines the ability of suppressive motifs to specifically down-regulate CpG-induced immunity.

Previous studies established that CpG DNA interacts with TLR9 to trigger the translocation of NF-κB from the cytoplasm to the nucleus and the subsequent up-regulation of cytokine gene expression (1, 6, 27–30). Current results demonstrate that this is not an all-or-none phenomenon. Although NF-κB translocation is initiated within minutes of CpG administration (29), the subsequent increase in cytokine production occurs over a period of hours (2) and is significantly reduced by the addition of suppressive ODN or the removal of stimulatory CpG DNA (Figs. 3 and 4). Consistent with these findings, suppressive motifs were recently shown to down-regulate CpG-dependent NF-κB and AP-1 induction (17, 18). These observations suggest that CpG motifs must continuously signal receptive cells for triggering to persist.

The sequence and length of a DNA strand determine its activity. By synthesizing and testing scores of ODNs, our laboratory and that of Krieg et al. independently identified G- and GC-rich motifs that selectively block CpG-dependent activation (16). Of note, Zhao et al. (31) showed that not all GC-rich repeats confer suppressive activity, while Halpern et al. (32) showed that ODNs containing runs of >15 polyGs can inhibit both CpG- and mitogen-induced immune responses. Suppressive activity appears to depend upon an ODN's secondary/tertiary structure, although sequence-nonspecific competition for ODN uptake is also possible (28). In this context, G-rich regions facilitate the formation of complex intra- and interchain Hoogsteen hydrogen bonds (33, 34). Depending on how these chains fold, activity may be gained or lost.

To validate the findings in this report, all experiments were repeated with multiple ODNs containing different combinations of suppressive and/or CpG motifs. In addition, the critical role of the suppressive motifs was established by showing that control motifs neither enhanced nor prevented CpG induced immune stimulation.

Table III. *Effect of distance between motifs on ODN activity*[a]

| ODN | Cytokine-Producing Cells (% maximum) | | |
|---|---|---|---|
| | IL-6 | IL-12 | IFN-γ |
| CpG ODN[b] | 100 ± 11 | 100 ± 7 | 100 ± 10 |
| CpG ODN[b] + Cont ODN | 97 ± 14 | 98 ± 9 | 100 ± 17 |
| CpG ODN[b] + Sup ODN | 16 ± 6 | 21 ± 6 | 18 ± 5 |
| LPS | 100 ± 2 | 99 ± 2 | 100 ± 2 |
| LPS + Sup ODN | 94 ± 7 | 94 ± 5 | 92 ± 7 |
| [CpG-Sup] ODN[b] | 87 ± 12 | >100 ± 14 | 92 ± 14 |
| [CpG-5 bases-Sup] ODN[b] | >100 ± 4 | >100 ± 21 | >100 ± 22 |
| [CpG-10 bases-Sup] ODN | 38 ± 6 | 64 ± 15 | 42 ± 7 |
| [CpG-20 bases-Sup] ODN | 7 ± 4 | 48 ± 13 | 24 ± 8 |
| [CpG-Cont] ODN[b] | 94 ± 7 | >100 ± 14 | 99 ± 11 |
| [Sup-CpG] ODN | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| [Sup-20 bases-CpG] ODN | 8 ± 5 | 9 ± 3 | 2 ± 1 |
| [CpG-Sup] ODN[b] + CpG ODN[b] | >100 ± 16 | >100 ± 15 | 98 ± 13 |
| [CpG-5 bases-Sup] ODN[b] + CpG ODN[b] | >100 ± 18 | >100 ± 11 | 98 ± 20 |
| [CpG-10 bases-Sup] ODN + CpG ODN[b] | 58 ± 7 | 75 ± 9 | 66 ± 9 |
| [CpG-20 bases-Sup] ODN + CpG ODN[b] | 27 ± 5 | 26 ± 10 | 30 ± 8 |
| [Sup-CpG] ODN + CpG ODN[b] | 9 ± 4 | 11 ± 4 | 8 ± 5 |
| [Sup-20 bases-CpG] ODN + CpG ODN[b] | 5 ± 1 | 9 ± 3 | 13 ± 2 |

[a] BALB/c spleen cells were stimulated in vitro with 1 μM of each ODN (or 5 μg/ml LPS), and the number of cells activated to secrete cytokine was determined 8 h later by ELISPOT. The percentage of cells activated to secrete cytokine was calculated by the formula: (number of cells activated by test ODN) − (background)/(number of cells activated by CpG ODN) − (background) × 100%. Multiple combinations of the CpG, suppressive and control 10-mer motifs described in Table II were used in these studies and gave similar results in these experiments. Results represent the average of two to four assays per data point. Table II shows typical numbers of cytokine-secreting cells per $10^6$ cells.

[b] Stimulatory ODN, $p < 0.05$.

The data in Tables II and III and Fig. 6 suggest that suppressive and stimulatory motifs are active on the same cells, and that their relative locations on a DNA strand determine the magnitude and nature of the resultant response. The results indicate that 1) cellular recognition of stimulatory and suppressive motifs proceeds in a 5′→3′ direction; and 2) suppression is generally dominant over stimulation, however, 3) when a CpG motif is immediately 5′ to a suppressive motif, stimulation dominates. A likely explanation for the latter phenomenon is that molecules involved in recognizing the 5′ motif block the cell's ability to interact with an immediately adjacent suppressive motif, perhaps due to steric hindrance. When the distance between motifs exceeds 10 bases, this effect dissipates.

Our finding that the relative location of CpG vs suppressive motifs on a single strand of DNA influences the resultant immune response strongly suggests that individual cells recognize both motifs. Experiments using labeled ODNs demonstrate that both types of DNA enter the same cells (Fig. 5 and data not shown). Indeed, the possibility that one type of cell responds only to stimulatory motifs and another only to suppressive motifs is inconsistent with the results in Tables II and III. Moreover, the data shown in Table I indicate that cells exposed to suppressive ODNs do not produce factors or interact on a cell-to-cell basis in such a way as to inhibit other cells from responding to CpG motifs.

Suppressive ODNs could be of use in several therapeutic settings. CpG motifs in antisense and gene therapy vectors contribute to the immune recognition of transfected cells (35). Introducing suppressive sequences 5′ to CpG motifs in these vectors might dampen this immune response and prolong the vector's in vivo activity (16). Alternatively, the immunogenicity of DNA vaccines might be improved by deleting suppressive motifs (16). Finally, suppressive ODN may prove useful in situations where the host's response to bacterial DNA contributes to pathology, as in septic shock or autoimmune disease (11, 25, 36, 37). Since suppressive ODN precisely target the inflammatory response induced by CpG DNA, these therapies may avoid the deleterious side effects associated with generalized immunosuppressive regimens.

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