

Heterogeneity in the Human Response to Immunostimulatory CpG Oligodeoxynucleotides

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Summary: Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs trigger an immune response characterized by proliferation, B-cell activation, and cytokine production. CpG ODNs show promise in animal models as vaccine adjuvants and anti-allergens and for the treatment of infectious diseases and cancer. Several different CpG motifs capable of stimulating human cells have been identified, and several CpG-containing ODNs are now in various stages of clinical trials. However, little is known of the comparative magnitude or breadth of the immune responses these CpG ODNs elicit. This work compares the proliferative, IgM, and IL-6 response of PBMCs from 100 normal donors to a panel of 11 ODNs selected as highly active from over 100 phosphorothioate ODNs. While PBMCs from every donor responded to at least one member of the ODN panel, no single ODN was optimally stimulatory in all subjects or for all responses. A mixture of ODNs with complementary activities induced significantly broader stimulation of donor PBMCs than any single ODN, providing a rational basis for proceeding into clinical trials with such a mixture. **Key Words:** CpG oligonucleotides, human PBMC, immune response, heterogeneity, cytokines, immunoglobulin

Bacterial DNA contains immunostimulatory CpG motifs that induce B cells, natural killer (NK) cells, and plasmacytoid dendritic cells to proliferate, mature, and secrete a variety of cytokines, chemokines, and/or Ig.^{1–4} Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs mimic the activity of bacterial DNA.^{3–6} Murine models indicate that these ODNs may be useful as vaccine adjuvants and anti-allergens and in the treatment of infectious diseases and cancer.^{7–10} However, the CpG motifs most active in mice are poorly stimulatory in primates due to evolutionary divergence in CpG recognition between species.^{11–13}

Several groups have identified CpG ODNs capable of stimulating human PBMCs to proliferate and secrete Ig and/or cytokine.^{11–14} These ODNs express multiple CpG

motifs on a phosphorothioate backbone. The ODNs studied by these different groups varied with respect to the number, location, and flanking sequence of the CpG dinucleotides they contain, and preferentially activated different immune cell types.^{11–13} Several of these ODNs are in clinical trials for a variety of applications, from allergy to cancer.

To help identify ODNs with the broadest activity to develop for human use, we evaluated the response of PBMCs from a large and heterogeneous population of donors to more than 100 CpG ODNs. We found that although PBMCs from all donors responded to at least one member of the ODN panel, no single ODN was universally active. Moreover, individual ODNs rarely induced optimal proliferation and IgM and IL-6 production in the same individual. Based on the response pattern of multiple subjects, a mixture of ODNs was identified that stimulated a significantly greater fraction of donor samples than any individual ODN. This mixture may be therapeutically superior for applications in which mul-

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tiple immune responses must be elicited in a diverse patient population.

MATERIALS AND METHODS

Cells

Normal PBMCs were obtained from the NIH Department of Transfusion Medicine. The characteristics of the donor population are shown in Table 1. Our studies evaluated the responses of approximately 20% of this donor pool. Mononuclear cells were isolated by density gradient centrifugation over Ficoll-Hypaque as described.¹³ Cells were cultured for 72 hours in RPMI supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine.

Reagents

ODNs were synthesized at the CBER Core Facility. All ODNs contained less than 0.1 EU/mg of endotoxin as measured by the *Limulus* amoebocyte lysate. Sequences of the phosphorothioate ODNs used commonly in this study are shown in Table 2.

ELISA Assays

Five × 10⁵ PBMCs were stimulated in vitro for 72 hours with 1 µmol/L ODN. IgM and IL-6 from culture supernatants were detected by ELISA as previously described.¹³ ELISA results were quantitated using standard curves generated using recombinant IL-6 and purified IgM. The limit of detection of the assays was 5 to 20 pg/mL. If cytokine/Ig production was below assay sensitivity, the lower limit of detection was used to calculate the stimulation index. All assays were performed in triplicate. ODNs were considered stimulatory if the response they induced exceeded that of unstimulated PBMCs from

TABLE 1. Characteristics of the PBMC donor pool

Characteristic	Percent of donor population
Sex	
Male	72
Female	28
Race	
White	70
Black	28
Hispanic	2
Age (years)	
Range	23–66
Average	40 ± 11

TABLE 2. Oligodeoxynucleotide sequences

Name	Sequence
CpG ODN	
K3	A T <u>C</u> G A C T C T <u>C</u> G A G <u>C</u> G T T C T C
K19	A C T C T <u>C</u> G A G <u>C</u> G T T C T C
K23	T <u>C</u> G A G <u>C</u> G T T C T C
K82	A C T C T G G A G <u>C</u> G T T C T C
K83	A C T C T <u>C</u> G A G G G T T C T C
K84	A C T C T <u>C</u> G A G C G T T C T A
K85	C A T C T <u>C</u> G A G <u>C</u> G T T C T C
K89	A C T C T T T <u>C</u> G T T C T C
K109	T <u>C</u> G A G <u>C</u> G T T C T
K110	T <u>C</u> G A G C T T C T C
K123	T <u>C</u> G T T <u>C</u> G T T C T C
Control ODN	
K121	A C T C T T G A G T G T T C T C

CpG dinucleotides are underlined.

that subject by more than fivefold. This minimized the effect of sequence-independent background stimulation associated with the use of phosphorothioate ODNs.^{15–17}

Proliferation Assays

One × 10⁵ PBMCs were stimulated for 72 hours with 1 µmol/L ODN. For the last 4 hours, 1 µCi ³H-thymidine was added to the cultures. Incorporated label was quantitated using liquid scintillation. All assays were performed in triplicate.

Statistical Analysis

The z test was used to compare the percent of donor samples responding to ODN mixtures versus individual ODNs. Nonparametric analysis of variance (ANOVA) was used to compare differences in the magnitude of the response induced by the mixture versus each of its components.

RESULTS

Response of Human PBMCs to the CpG ODN Panel

CpG ODNs that activate human PBMCs vary in the number, location, and flanking regions of the CpG dinucleotides they contain.^{11–13} To compare the activity of different ODNs, their ability to stimulate PBMCs to proliferate and to produce IgM and IL-6 was examined.¹³ Based on preliminary dose-response studies, and considering that non-CpG ODNs containing a phosphorothioate backbone have some immunostimulatory properties, CpG ODNs were evaluated in all three assays at a concentration of 1 µmol/L. At this concentration CpG ODNs

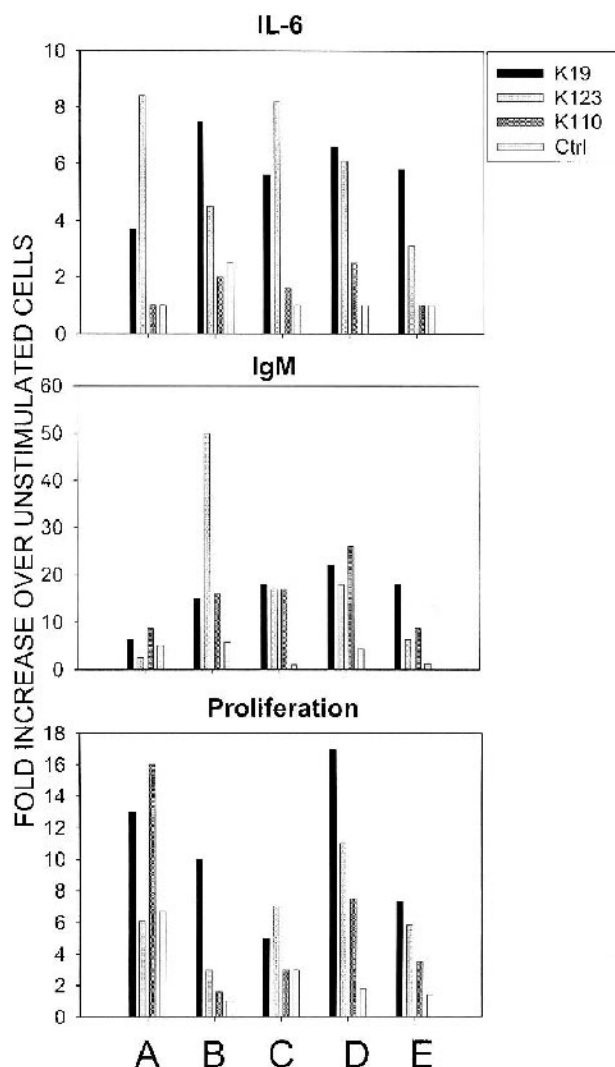


FIGURE 1. PBMCs were incubated with 1 μ mol/L of various CpG or control ODNs (sequence 5'-TGCAGGCTTCTC-3'). Fold inductions over media for proliferation, IL-6, and IgM are shown for five different donors. Intra-assay variability was <10%.

were significantly more active than control, non-CpG, ODNs (data not shown).

Initial experiments (Fig. 1) compared responses of PBMCs from five donors treated with three CpG and one control (non-CpG) ODNs. Unexpected heterogeneity in the response of donors to this ODN panel was observed. While for donor A, K19 and K110 induced weak IgM production and strong cell proliferation, donor C responded equally to all three CpG ODNs for IgM production but only to K19 and K123 for IL-6 production. In donor B, K19 surpassed the other CpG ODNs at inducing IL-6 production and proliferation, while K123 triggered optimal IgM production. All of the CpG ODNs tested triggered all three types of response in donor D. In donor

E, K19 was the most stimulatory ODN in all three assays, while K110 failed to stimulate a significant IL-6 response. These findings suggested that ODNs that elicit an optimal response in one individual might be suboptimal or even inactive in another individual. Moreover, an ODN that strongly stimulates one type of immune response may fail to trigger a different immune parameter in the same individual. To confirm these conclusions, a larger population of donors was evaluated.

Over 100 CpG ODNs were screened for their ability to stimulate PBMCs from a diverse pool of normal donors (see Table 1). Eleven of the most stimulatory ODNs (Table 2) were then tested for their ability to stimulate IL6, IgM, proliferation, or all three responses simultaneously, on PBMCs from over 100 donors. ODNs K3, K19, and K23 were the most effective at inducing proliferation, stimulating significant (>5-fold) proliferation by more than 90% of donor samples (Table 3). Each of these ODNs contains at least two different CpG motifs (including CTCGAG and AGCGTT). By comparison, ODNs K83 and K89 (which express a single CpG motif) failed to trigger over a third of the PBMC samples to proliferate. Control ODN stimulated a response in only 21% of the donor samples, and the magnitude of the response was significantly lower ($P < 0.05$) compared with CpG ODNs (see Table 3, and data not shown). Interestingly, none of the CpG ODNs studied was able to stimulate cells from every donor to proliferate.

IL-6 and IgM production was also analyzed. Greater than 80% of donor samples were stimulated to secrete IL-6 by ODN K3 (see Table 3), which, as noted above,

TABLE 3. Percent of donor samples responding to stimulation by individual ODNs

ODN	Proliferation	IL-6 production	IgM production	All
K3	93 (54)	82 (50)	59 (49)	62 (38)
K19	92 (76)	52 (61)	88 (56)	48 (42)
K23	93 (44)	71 (45)	55 (33)	64 (28)
K82	88 (8)	56 (9)	89 (9)	63 (8)
K83	63 (8)	67 (9)	78 (9)	63 (8)
K84	67 (9)	73 (11)	89 (9)	63 (8)
K85	56 (9)	73 (11)	89 (9)	60 (8)
K89	57 (7)	67 (9)	57 (7)	50 (6)
K109	67 (9)	64 (11)	71 (7)	57 (7)
K110	81 (68)	36 (58)	60 (53)	27 (41)
K123	90 (70)	52 (54)	65 (55)	43 (46)
K121 (non-CG)	21 (68)	11 (47)	35 (49)	3 (39)

The percent of donor samples with significantly elevated responses to each ODN (exceeding media alone by >5-fold) is shown. The number of samples tested for each condition is indicated in parentheses. The percent of subjects responding (>5-fold) in all three assays is provided in the final column. Not all PBMC samples were tested in all assays, and these were not included in the "All" analysis.

expresses multiple different CpG motifs. ODNs containing multiple CpG motifs were generally more active at inducing IL-6 production than those with only one motif (compare K3 to K89). There was no discernible pattern for IgM secretion with respect to the length or number of CpG motifs contained in an ODN, but the phosphorothioate backbone contributes considerably to IgM induction, since many individuals responded to the non-CpG ODN. Importantly, there was some bias in the type of stimulation elicited by certain ODNs. For example, K110 was one of the strongest inducers of proliferation but was relatively weak at stimulating IgM or IL-6 secretion, whereas K3 was a potent inducer of proliferation and IL-6 production, but not IgM secretion.

To examine the breadth of the stimulatory response elicited by individual ODNs, their ability to simultaneously trigger at least a fivefold increase in the response for all three immune parameters (proliferation and IgM and IL-6 production) was examined. Whereas some ODNs were very effective at inducing one or two responses, no single ODN elicited all three types of response in greater than 64% of the donor pool. For example, K3 stimulated 92% of the PBMC samples to proliferate and 82% to produce IL-6 but triggered only 59% of the same donors to produce IgM. The control ODN elicited a comprehensive response in only 3% of the subjects.

To determine whether assay variability contributed to this heterogeneity, the response of freshly isolated PBMCs from the same donors was monitored on multiple occasions. An individual's pattern of reactivity to each ODN was reproducible, indicating that the observed heterogeneity was not due to assay variability (data not shown).

Several novel insights are provided by this series of studies. Although PBMCs from all donors responded to at least one member of the ODN panel, no single ODN activated PBMCs from all donors in all assays. Different ODNs were frequently required for the induction of optimal IL-6 and IgM and proliferative responses by the same subject. ODNs containing multiple CpG motifs were generally more active than those containing a single motif for stimulating proliferation and IL-6 production.

ODN Mixtures are Broadly Immunostimulatory

To overcome the limitations associated with the use of individual ODNs, mixtures were prepared from ODNs selected for their strong activity on PBMCs from different subjects in different assays. Preliminary studies showed that mixtures composed of three ODNs were optimally effective (data not shown). Several such mix-

tures stimulated a significantly broader pool of donor PBMCs in more assays than any single ODN (Table 4). The most effective mixture (composed of 0.33 $\mu\text{mol/L}$ each of K3, K19, and K110, yielding a composite ODN concentration of 1 $\mu\text{mol/L}$) stimulated 94% of donors to proliferate and secrete IgM and IL-6. This significantly exceeded the breadth of the activity elicited by any single ODN at a concentration of 1 $\mu\text{mol/L}$ ($P < 0.05$, Tables 3 and 4). Thus, this mixture of ODNs exceeded the ability of individual ODNs to trigger a diverse population of donors to mount a broad range of immune responses.

To examine the magnitude of the stimulation elicited by this mixture, the response induced by K3/K19/K110 was compared with each of its components on 11 donors. Dose-response curves demonstrated that 1 $\mu\text{mol/L}$ of CpG ODN elicited an optimal response (data not shown). In this study, individual ODNs were used at a concentration of 1 $\mu\text{mol/L}$ while each component of the mixture was used at 0.33 $\mu\text{mol/L}$, yielding a final concentration of 1 $\mu\text{mol/L}$. On average, the mixture stimulated a 13-fold increase in proliferation, a 6-fold increase in IL-6 production, and an 11-fold increase in IgM secretion (Fig. 2). The response to the mixture did not differ significantly from the stimulation induced by the most active single ODN in each of these assays ($P > 0.05$).

DISCUSSION

Murine studies suggest that CpG ODNs are useful as immunotherapeutic agents.^{7-10,18-20} However, ODNs that are highly active in mice are poorly immunostimulatory in man.¹¹⁻¹³ Considerable effort has been invested in identifying ODNs capable of activating human cells, and several groups have identified such human-active CpG ODNs. The described CpG ODNs vary dramatically in sequence and structure as well as CpG number and placement. Although some CpG ODNs are in clinical trials, no systematic studies thus far have addressed heterogeneity in the human response to CpG ODNs. This work compares the ability of 11 highly active ODNs

TABLE 4. Percent of donor samples responding to stimulation by ODN mixtures

ODN mixture			Proliferation	IL-6	IgM	All
3	19	110	96 (25)	95 (19)	91 (23)	94 (17)
19	23	123	100 (17)	86 (14)	100 (14)	83 (12)
3	110	123	94 (17)	93 (15)	87 (15)	77 (13)
3	23	123	95 (20)	78 (18)	89 (18)	67 (15)
3	9	123	95 (20)	67 (18)	83 (18)	53 (15)
19	110	123	77 (31)	50 (20)	82 (22)	35 (17)

The percent of donor samples responding by proliferating or producing IL-6 or IgM or all three responses (All) is shown. The number of samples tested for each condition is shown in parentheses.

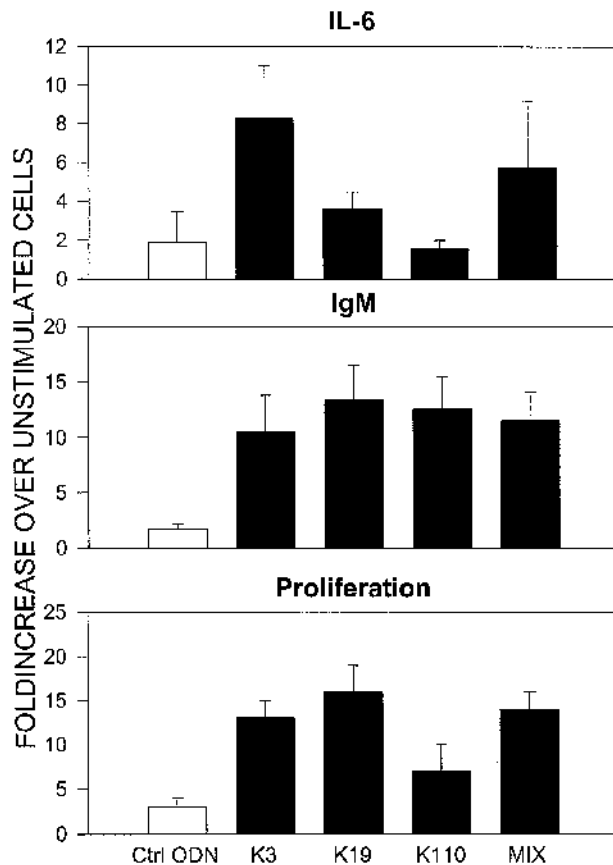


FIGURE 2. PBMCs from 11 donors were stimulated with 1 $\mu\text{mol/L}$ of individual CpG ODN, or a mixture of K3, K19, and K110 containing 0.33 $\mu\text{mol/L}$ of each component for 72 hours. The magnitude of the proliferative, IL-6, and IgM responses was determined for each subject. Overall, the magnitude of the response to the mixture was equivalent to that of the maximally stimulatory single ODN in all assays ($P > 0.05$).

selected from a panel of more than 100 sequences to stimulate IgM, IL-6, and proliferative responses in PBMCs from a large and diverse pool of normal donors. Results indicate that the human response to CpG ODNs is heterogeneous. No single ODN was stimulatory in all donors, and optimal Ig, cytokine, and proliferative responses were commonly elicited by different ODNs, even in the same donor. A mixture was identified that induced a broader response in a greater fraction of donors than any single ODN, suggesting that a combination of ODNs may be more effective for clinical application than any single ODN.

Data from previous studies support the conclusion that the human response to CpG ODNs can be heterogeneous. In a report by Bohle et al, PBMCs from only 44% of subjects mounted a significant (>5 -fold) TNF α response when stimulated with ODN K3.²¹ Similarly, the response of PBMCs from a small group of donors

showed considerable variability when stimulated with a panel of ODNs in a study conducted by Hartmann et al.²² We previously demonstrated that different types of CpG ODNs (varying in CpG motif and backbone composition) preferentially activated different cell types.¹³ Yet the above studies were undertaken to identify CpG motifs with activity in humans and were not designed to detect or quantify intersubject variability. To overcome this limitation, the current work compared the breadth and magnitude of multiple immune responses elicited by a large panel of ODNs in over 100 normal donors. This approach established that the human response to CpG ODNs is unequivocally heterogeneous.

There are several potential sources for this heterogeneity. Since CpG ODNs activate a variety of immune cell types,¹³ heterogeneity between individuals may reflect differences in the relative frequency of each cell type as well as the reactivity of each cell population to specific CpG motifs. That is, if B cells respond better to K3 than K19, subjects who have higher number of B cells might show a stronger response to K3. Future studies addressing both the response of different cell populations to different ODNs and ratios of these populations in PBMCs from different donors should shed light on the impact of these factors on the observed heterogeneity. In addition, although the innate immune system is not usually considered to have “memory” in the sense that the adaptive immune system has, exposure of an individual or a population to CpG DNA (from pathogens or vaccines) may over time shape their subsequent response to specific DNA sequences.

Superimposed on this subject-to-subject variability may be population-based differences in CpG recognition. For example, pathogens endemic to distinct geographic locations may express different CpG motifs, thus exerting selective pressure leading to divergence in CpG recognition by inhabitants of those locations. Consistent with such a possibility is the finding that CpG recognition by rodents and primates has diverged over evolutionary periods. This is reflected by differences at both the nucleotide and amino acid sequence level in the CpG receptor TLR 9.^{23–25} Our laboratory is examining whether polymorphisms in TLR 9 contribute to variability in the human response to CpG ODNs. Experiments are also underway to evaluate whether genetic and/or environmental factors influence a donor’s ability to recognize specific CpG motifs. Available data on 34 subjects provides no evidence that race, gender, or age correlates with the response to specific CpG ODNs. Other receptors on the surface of B cells have also been described to

contribute to the CpG ODN response;¹⁵ therefore, polymorphisms in this or other yet unidentified co-receptors may also be responsible for the heterogeneity in the response to CpG ODNs described in this paper. However, the heterogeneity in the response described in this paper was not due to interassay variation, since the individual pattern of response to CpG ODNs was reproducible in subjects tested on multiple occasions with the same sequences (data not shown).

ODNs with phosphorothioate backbones are known to have sequence-nonspecific stimulatory effects on PBMCs. The magnitude of this response is also heterogeneous but rarely exceeds background by more than fivefold. Care was taken to minimize these sequence-nonspecific effects by studying ODNs at the low fixed concentration of 1 $\mu\text{mol/L}$, and responses were considered positive only if they exceeded background by more than fivefold (see Fig. 1). We recognize that this strategy underestimates the activity of ODNs that are optimally stimulatory at lower concentrations. Comparing CpG to non-CpG ODNs, instead of to untreated, does not change the outcome of this study; however, the stimulatory response was compared with that of unstimulated cells, since current clinical trials compare serum cytokine levels between individuals injected with CpG ODNs to those injected with diluent alone. LPS is not likely to be a source of non-CpG-specific stimulation, since CpG and control ODNs were synthesized in parallel in the same facility, maintained under sterile conditions, and repeatedly found to be endotoxin-free and not to elicit TNF α secretion by PBMCs in culture.

A critical finding from the current work is that a mixture of ODNs is more broadly active than any single ODN. Each component of the optimally stimulatory mixture identified in this study expressed a different CpG motif at its 5' end, the site of greatest immunostimulatory activity. It is interesting that the presence of a less stimulatory CpG ODN did not undermine the effect of the optimal CpG ODN. Rather, the effect of the multiple ODNs at suboptimal concentrations appears to be additive. Ongoing basic studies should determine whether a sufficiently complex ODN capable of triggering PBMCs from all donors in all assays can be synthesized, or whether a mixture of less complex ODNs will provide the greatest therapeutic benefit in humans. The fact that individual CpG ODNs lack activity on the entire population and that CpG ODN mixtures are active on a broader fraction of the population suggests that CpG ODN mixtures may have an improved therapeutic effect and should be tested in future clinical trials.

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