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Control of In Vitro Immune Responses by Regulatory Oligodeoxynucleotides through Inhibition of pIII Promoter Directed Expression of MHC Class II Transactivator in Human Primary Monocytes

Jinhai Wang,1* Gregory Roderiquez,* Taneisha Jones,* Peter McPhie,† and Michael A. Norcross*

Ag presentation is a key step in the initiation of adaptive immune responses that depends on the expression of MHC Ags and costimulatory molecules. Immune-enhancing CpG and non-CPG oligodeoxynucleotides (ODNs) stimulate Ag presentation by stimulating the expression of these molecules and by promoting dendritic cell maturation. In this report, we identify immunoregulatory orthophosphorothioate non-CpG molecules, referred to as regulatory ODNs (rODNs), by their ability to inhibit allogeneic monocyte-stimulated T cell responses and down-regulate HLA-DR in human primary monocytes. The rODNs promoted the survival of macrophages and were able to activate IL-8 secretion through a chloroquine-resistant pathway. Messenger RNAs for HLA-DR α and β and the MHC CIITA were reduced by rODNs but not by stimulatory CpG ODN2006 and non-CpG ODN2006a. CIITA transcription in monocytes was controlled primarily by promoter III and not by promoter I or IV. rODNs blocked promoter III-directed transcription of CIITA in these cells. Under conditions that induced dendritic cell differentiation, rODNs also reduced HLA-DR expression. The activity of rODNs is phosphorothioate chemistry and G stretch dependent but TLR9 independent. G tetrads were detected by circular dichroism in active rODNs and associated with high m.w. multimers on non-denaturing gels. Heat treatment of rODNs disrupted G tetrads, the high m.w. aggregates, and the HLA-DR inhibitory activity of the ODNs. The inhibition of immune responses by regulatory oligodeoxynucleotides may be useful for the treatment of immune-mediated disorders including autoimmune diseases and graft rejection. The Journal of Immunology, 2007, 179: 45–52.

P olymorphic HLA-DR proteins are encoded by MHC class II genes and expressed as αβ-heterodimers on the cell surface. These molecules play essential roles in the initiation of adaptive cellular and humoral immune responses. MHC class II molecules are involved in autoimmune disorders and transplant rejection. The constitutive expression of MHC class II proteins, unlike the MHC class I molecules that are universally expressed on most nucleated cells, is limited to APCs including macrophages, dendritic cells (DCs),2 B lymphocytes, and thymic epithelial cells. Expression of class II MHC genes necessary for exogenous Ag presentation is controlled by the CIITA. CIITA is the so-called “master control factor” for the expression of MHC class II genes (1, 2). The gene encoding CIITA is MHC2TA. Transcription of MHC2TA gene is driven by one of three alternative promoters, pI, pIII, or pIV (3). Alternative splicing of transcripts produces three isomeric mRNAs that differ in the first exon. These three isoforms differ in N-terminal sequences but share four common domains (acidic domain, proline/serine/threonine-rich domain, GTP-binding domain, and leucine-rich repeats) (2). MHC class II molecules are not detected on the surfaces of splenic B cells and DCs in CIITA knockout mice (2), indicating tissue-specific impairment of MHC class II expression.

CpG oligodeoxynucleotides (ODNs) have multiple effects on immune cells, including B cells, DCs, and monocytes/macrophages. Several different types of immune-enhancing ODNs have been described. Type A ODNs, such as ODN2216, are potent type I IFN inducers, whereas type B ODNs, such as ODN2006, are potent B cell stimulators with weaker IFN inducing ability (4). We previously observed that non-CpG ODNs based on type B CpG ODN2006, when mixed with GM-CSF, induced chemokine secretion and DC maturation with high levels of HLA-DR, CD86, CD40, and CD83 expression in human primary monocytes (5).

In contrast to immune-enhancing CpG ODNs, inhibitory CpG ODNs have also been reported that down-modulated MHC expression on ConA-activated murine macrophages (6). Agents that can down-modulate HLA-DR and thereby inhibit adaptive immune responses may provide novel treatments for graft rejection, graft-vs-host disease, autoimmune disease, and diabetes.

Inhibitory non-CpG ODNs that are able to down-modulate HLA-DR on human cells have not been reported. In this study we identify a class of phosphorothioate (PS) non-CpG ODNs, referred to as regulatory ODNs (rODNs), that inhibits the expression of CIITA and cell surface HLA-DR on monocytes and reduces monocyte-stimulated, allogeneic T cell proliferative responses.

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2 Abbreviations used in this paper: DC, dendritic cell; CD, circular dichroism; ODN, oligodeoxynucleotide; PO, phosphodiester; PS, phosphorothioate; rODN, regulatory ODN.

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Materials and Methods

Cells and reagents

Human monocytes and PBLs were isolated by countercurrent centrifugal elutriation from single-donor preparations of peripheral blood leukocytes as described previously (7, 8). Monocyte preparations contained <1% of plasmacytoid DCs as detected by anti-BDCA-4 Ab staining. Cells were cultured in macrophage serum-free medium (Invitrogen Life Technologies).

Oligodeoxynucleotides

The following ODNs were synthesized by the Center for Biologics Evaluation and Research Core Facility (Table I).

Cell surface immunofluorescence staining

Cells were stained with the indicated Abs to cell surface Ags and then subject to flow cytometry analysis as described previously (7, 9). Anti-HLA-DR, anti-CD86, and isotype controls were purchased from BD Pharmingen.

Chemokine secretion

The amounts of IL-8 secreted by monocytes into culture supernatants were measured using ELISA kits purchased from BioSource International. Data were analyzed with SoftMax Pro software.

RT-PCR analysis

Total cellular RNA of human monocytes was isolated using TRIzol reagent and then treated with DNase I. Equal amounts of total RNA were subject to first-strand cDNA synthesis using RNase H minus SuperScript II reverse transcriptase (Invitrogen Life Technologies). cDNA was amplified by 30 cycles of PCR and then treated with DNase I. Equal amounts of total RNA were subject to the following primers for GAPDH, 5'-GCCATGCCGAGCCTTGG 3' and 5'-GCCATGCCGAGCCTTGG 3'; type IV CIITA, 5'-GCTTGGGGCGCTTGAAGAATT-3' and 5'-ACCTTGAGCCCTCAAAGCTGGA-3'; type III CIITA, 5'-CAATGCTAGGTACTGCGGGAG-3' and 5'-CAATGCTAGGTACTGCGGGAG-3'; and type I CIITA, 5'-GAAGCTCCAGGTAGCCACCTTCTA-3' and 5'-GAAGCTCCAGGTAGCCACCTTCTA-3'. PCR products were separated and stained with SYBR Gold (catalog no. S11494; Invitrogen Life Technologies) nucleic acid gel stain and analyzed with a FluorImager scanning densitometer and ImageQuant software.

Circular dichroism (CD) analysis

Solutions of oligonucleotides (A260 = 1) were made up in PBS. CD spectra were measured by a Jasco J-715 spectropolarimeter with the solutions in a 1-cm pathlength quartz cuvette in a cell holder thermostated by a Neslab RTE-111 circulating water bath. Spectra were scanned four times, from 330 to 210 nm and averaged (speed 50 nm/min, time constant 1s). Spectra were obtained at 5°, 25°, and 70°. To study the effect of potassium ions, 1 M KCl solution was added to give a final concentration of 50 mM. After baseline correction, the measured ellipticities were converted into mean residue mdeg values using the formula \( \Delta \varepsilon = (100 \times mdegs) \times (330 \times c) \), where mdegs is the measured ellipticity, and c is the concentration of nucleotide residues (mM).

Results

Suppression of monocyte alloantigen-induced proliferation of T lymphocytes by rODNs

During the course of studies to identify immune modulatory effects of ODNs, we observed that some non-CpG oligonucleotides had suppressive effects on allogenic T cell responses. We therefore synthesized a series of non-CpG ODNs (all listed in Table I) and tested their activity on monocyte-activated allogeneic responses. Monocytes were treated overnight with this set of ODNs, mixed with allo-PBLs at a 1:2 ratio for 3 days, and cell proliferation was measured. As shown in Fig. 1, rODNs 0001 and 0002 inhibited T cell proliferation by 50% compared with the untreated controls. rODN0009 with GG and rODN0012 with GG, AA, and CC at the marked dinucleotides also showed inhibition of >50% (Table I, rODN0001, boldface). rODN0010 with two GG and one CC at the marked dinucleotides also showed inhibitory activity (Table I), rODN0005 and rODN0006, with replacement of the G-rich regions at both sides, showed weak inhibitory activity. Oligos rODN0003 and rODN0004, which retained one G stretch, remained active. rODN0007 and rODN0008, the phosphodiester (PO) versions of rODN0001 and rODN0002, respectively, did not show inhibition of the proliferative response. These results indicate that the marked sequences internal to the flanking G stretches were not critical to the activity and that at least one G stretch was required for inhibition. PS linkages were required for activity under these conditions.

Down-modulation of HLA-DR by rODNs

Trimolecular interactions of MHC peptide-TCR are the key steps in induction of Ag-specific immune responses. HLA-DR presents...
exogenous peptides to CD4+ T lymphocytes. We examined the effects of these rODNs on cell surface expression of MHC II molecules. As shown in Fig. 2, HLA-DR expression was reduced by 80% with rODN0001, rODN0009, and rODN0012, but not by rODN0006, which has C-substituted flanking G stretches. HLA-DR was also down-modulated by rODN0012 at 1 μg/ml (data not shown).

**Transcriptional inhibition of DRA and DRB by rODN**

HLA-DR is formed by products of the DRA and DRB genes. We next examined the impact of rODN on DRA and DRB messages in monocytes. Cells were treated with rODN00012 with and without stimulatory ODNs 2006 and 2006a. As shown in Fig. 3, DRA and DRB messages were highly expressed in untreated monocytes, but their expression was reduced in rODN0012-treated cultures. ODN 2006 and 2006a did not inhibit DR messages directly or interfere with rODN0012 inhibitory activity.

**Transcriptional blocking of CIITA**

The lack of CIITA in cells from the bare lymphocyte syndrome leads to the absence of MHC class II transcription by interfering with the function of the MHC enhancesome (11). To investigate whether rODN regulates HLA-DRA and HLA-DRB gene expression through the control of CIITA expression, the levels of CIITA mRNA in monocytes treated with rODN were examined 5 h after exposure to rODNs, a time point chosen based on the fact that the half-life for CIITA mRNA is 3.5 h for type I and 7.8 h for type IV (12). CIITA RNA was detected in cultured control monocytes and did not change with immune stimulatory CpG ODN2006 and non-CpG ODN2006a treatment. CIITA RNA was almost completely blocked in rODN0012-treated monocytes, indicating that rODN may regulate MHC class II molecules by limiting CIITA levels. Again, ODN2006 and ODN2006a did not induce CIITA directly or interfere with rODN0012 inhibition of CIITA when cocultured with the inhibitory oligonucleotides.

**Inhibition of pIII promoter activity by rODN**

There are at least three independent promoters controlling human CIITA transcription (3). pI directs the constitutive expression of CIITA in DCs and B lymphocytes. pIII is used for the constitutive expression of CIITA in DCs and B cells and for the induction of CIITA in activated T cells (3, 13, 14). pIV is predominantly induced by IFN-γ (15). We first determined which promoter...
was used in monocytes by RT-PCR amplification of the first exon of each promoter and found that CIITA expression was exclusively driven by the pIII promoter in human primary monocytes (Fig. 4A). We then examined the impact of rODNs on pIII promoter-directed CIITA RNA expression and found that pIII-directed expression of CIITA was dramatically blocked by rODN0012 (Fig. 4B).

**Reduction of HLA-DR expression in DCs**

Monocyte-derived DCs that express TLR9 are generated in the presence of IL-4 and GM-CSF (16). We have reported that elutriated monocytes differentiate into mature DCs after stimulation with GM-CSF and PO non-CpG ODNs, but not with PO ODNs as seen by induced expression of CD83, HLA-DR, and CD86 (5). To study the impact of rODN on DC maturation, we cultured monocytes with rODN in the presence of the DC maturation stimulators GM-CSF and non-CpG ODN 2006a and then measured the expression of HLA-DR and CD86. Adding rODN0001 at 10 μg/ml reduced HLA-DR levels without substantially affecting CD86 expression (Fig. 5A) in activated monocytes, indicating that rODN affects DC differentiation and maturation by selectively reducing class II molecules. A 10-fold lower dose of rODN also inhibited HLA-DR expression under these conditions (Fig. 5B).

**G-stretch dependent down-modulation of HLA-DR**

To test whether the sequences between the two G-stretches are important for down-modulation of HLA-DR, eight new oligos were synthesized and tested. As shown in Table II, rODN0013, 0014, and 0015 were inhibitory in cells treated with GM-CSF and ODN2006a. rODN0016 and 0017, in which all sequences between the two G stretches were replaced with either A or T, were also inhibitory, indicating that the inhibitory activity is not dependent on the sequences between the two G stretches. We then determined whether the spacing between the two G stretches is essential for the inhibitory activity. Both rODN0018 and 0019 have shorter spacing between the two G stretches, but both maintained down-modulating activity. It was further shown that rODN0020, which has the same base composition as 0012 with one G stretch at the left end as in 0012 but with the other G stretch placed in the middle of the oligo (Table II), and 0021, which only has one five G stretch in

### Table II. Inhibition of HLA-DR expression by rODNs

<table>
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<tr>
<th>rODN</th>
<th>Sequences</th>
<th>Percent Inhibition (%)</th>
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*Monocytes were cultured in serum-free medium with GM-CSF and non-CpG ODN2006a.*

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**FIGURE 6.** Regulation HLA-DR expression by PS ODNs consist of pure G stretches. Monocytes were treated overnight with indicated ODNs in the absence or presence of GM-CSF and ODN2006a (A) or in the presence of GM-CSF and ODN2006a or ODN2006 (B) and then stained with the indicated Ab and subjected to FACS analysis. Med, Medium.
the middle of the rODN (AGGATGGGGGAATCC), also down-modulated HLA-DR (data not shown). These results indicate a dependence on G-stretches for HLA suppression and are consistent with the G dependence of rODN inhibitory effects on allo-responses.

To test whether a G stretch is the principal activity domain, PS ODNs with 20 G and five G nucleotides were synthesized and tested on human monocytes. As shown in Fig. 6A, both 20-G ODNs and 5-G ODNS down-modulated HLA-DR expression in monocytes in the absence or presence of the DC differentiation/maturation factors GM-CSF and non-CpG ODN2006a, indicating that G stretches are the major activity domain of regulatory ODNs. To define the minimal number of G nucleotides in a sequence required for inhibition we tested GGGGG, GGGGGC, and GGGGCC oligonucleotides on HLA-DR expression on resting and activated human monocytes. HLA-DR was inhibited in both stimulated and unstimulated cells by GGGGG and GGGGCC (Fig. 6B), thus supporting the notion that a five G nucleotide sequence is the minimal functional domain. In the contrast, HLA-DR was not down-modulated by the PS ODNs TTTTT, CCCCC, and AAAAA (data not shown), indicating that the HLA-DR down-modulating activity is poly(G) specific.

**rODNs stimulate chemokine CXCL8/IL-8 production in monocytes through a chloroquine-insensitive pathway**

Although rODNs suppressed cell surface HLA-DR, it was not clear whether these rODNs were globally suppressive or had activating effects on other functions. We next tested whether rODNs had direct effects on another inflammatory secretory product, the chemokine IL-8. Monocytes were treated with rODN0001 or with the non-CpG ODN2006a in the presence or absence of chloroquine, an endosome maturation/acidification inhibitor. Endosome acidification is required for CpG ODN activity (17) and for the optimal induction of CCL3 by non-CpG ODN derived from type B CpG ODN2006 (5). Cells were precultured with chloroquine and then stimulated with rODN and ODNs. We found that IL-8 was induced by rODN0001 and ODN2006a. However, only ODN2006a-induced but not rODN0001-induced IL-8 production was inhibited by chloroquine (Fig. 7), indicating that the mechanisms of induction of IL-8 by rODN and ODN2006a are different.

**Multimer formation of active regulatory ODNs**

To examine whether regulatory ODNs exist as multimers, rODNs were analyzed by using PAGE with a highly sensitive staining method for DNA. In addition to the main single-stranded bands, all rODNs formed heterogeneous high m.w. forms or multimers except for rODN0005 (Fig. 8A) and rODN0006 (data not shown), indicating that the G stretch is essential for the formation of multimers.

**Multimers and regulatory activities are heat-sensitive**

Previous studies showed that aggregated formation and functional activities of these G-rich ODNs are heat sensitive (18). To test whether the multimers of rODNs are labile to heat, rODN0005, 0009, 0012, and 0017 were heated for 10 min at 90°C and analyzed by using PAGE. rODNs 0009, 0012, and 0017 all formed multimers that were disrupted with heating (Fig. 8A).

We then examined whether the activity of rODNs was sensitive to heat. As shown in Fig. 8B, after heating rODN 0012 lost its activity in the down-modulation of HLA-DR.

**G stretch-dependent formation of G tetrads**

G-rich sequences can form several kinds of secondary structures, including unimolecular hairpins or multistranded structures that may involve non-Watson-Crick G-G base pairs. Potassium ions can stabilize these multistranded G quadruplex structures. These structures can be identified by their characteristic CD spectra (19). Fig. 9A shows the CD spectra of ODN0005 and ODN0006, which do not contain G stretches and are inactive in decreasing...
HLA-DR expression. Positive peaks at 280 nm and small negative ellipticities below 260 nm indicate that these oligonucleotides are single stranded. In contrast, the spectra shown in Fig. 9B show that the G stretch-containing ODNs rODN0001, rODN0002, and rODN0012 all form multistranded structures. The intense positive peak at 260 nm, shown by all of these nucleotides, indicates the presence of extensive amounts of parallel G quadruplexes. The variable shoulders near 300 nm

FIGURE 9. Structure analysis of rODNs by CD. CD analysis of regulatory ODNs in solution was performed as described in Materials and Methods. A, rODN0005, and rODN0006. B, rODN0001, rODN0002, and rODN0012. C, rODN0012 at 25°C and 70°C.
produced inflammatory TNF-α production by murine splenocytes was inhibited by an ODN with only size exclusion analysis. In this report we show that MHC class II containing ODNs also formed large m.w. heterogeneous populations which was shown to correlate with four stranded helices stabilized tetrad formation. Poly(G) binding to the macrophage scavenger receptor are inhibited by poly(G) ODNs (29). Poly(G) ODNs induced the expression of TLR9 in elutriated monocytes (5), which may increase monocyte responses to CpG and non-CpG ODNs. Recent studies indicate that TLR9 is also the receptor for certain non-CpG ODNs (43, 44). In accordance with these findings, we reported that GM-CSF to some extent derived from type B CpG ODN2006 is endosome acidification/maturation dependent (5). However, a recent study indicated that suppressive ODNs inhibit LPS-mediated endotoxic shock by direct binding to STAT1 and STAT4 in macrophages (23) and that B-form DNA induces antiviral responses in a TLR-independent manner (36). In the current study, we found that rODNs that contain a G-rich PS backbone with no CpG motifs strongly activate IL-8 secretion while inhibiting HLA expression. Activation of IL-8 production was not sensitive to pretreatment with chloroquine in contrast to non-CpG stimulatory ODNs, supporting a non-TLR9 dependent signaling pathway for rODN effects. DCs are the most potent APCs (46). Monocytes become immature DCs after migrating from peripheral blood into tissues. After capturing Ags these immature DCs mature and migrate to lymph nodes where they present captured Ags as peptide fragments to T cells and stimulate T cell-dependent immunity. By inhibiting Ag presentation of monocytes and differentiation/maturation of monocytes to DCs, rODNs are possible candidates for attenuating immune responses. Inhibitory strategies incorporating peptides that control monocyte migration (47) along with rODN may be effective in controlling immune responses in vivo. Through the control of poly(G) stretches and the poly(G) motif, rODNs inhibit expression of the CD86 costimulatory molecule.