Impact of Secondary Structure of Toll-Like Receptor 9 Agonists on Interferon Alpha Induction[▽]

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Oligodeoxynucleotides containing a CpG motif and double- or multistranded structure-forming sequences act as agonists of Toll-like receptor 9 (TLR9) and induce high levels of interferon alpha (IFN- α) in addition to other Th1-type cytokines. In the present study, we evaluated three highly effective IFN- α -inducing agonists of TLR9 to determine the type of duplex structures formed and the agonist's ability to induce immune responses, including IFN- α induction, in human cell-based assays and in vivo in mice and nonhuman primates. Thermal melting studies showed that two of the agonists evaluated had a single melting transition with similar hyperchromicity in both heating and cooling cycles, suggesting the formation of intermolecular duplexes. A third agonist showed a biphasic melting transition in the heating cycle and a monophasic melting transition with lower hyperchromicity during the cooling cycle, suggesting the formation of both intra- and intermolecular duplexes. All three agonists induced the production of Th1-type cytokines and chemokines, including high levels of IFN- α , in human peripheral blood mononuclear cell and plasmacytoid dendritic cell cultures. Subcutaneous administration of the two intermolecular duplex-forming agonists, but not the intramolecular duplex-forming agonist, induced cytokine secretion in mice. In nonhuman primates, the two agonists that formed intermolecular duplexes induced IFN- α and IP-10 secretion. On the contrary, the agonist that formed an intramolecular duplex induced only low levels of cytokines in nonhuman primates, suggesting that this type of structure formation is less immunostimulatory in vivo than the other structure. Taken together, the present results suggest that oligonucleotide-based agonists of TLR9 that form intermolecular duplexes induce potent immune responses in vivo.

The vertebrate immune system recognizes highly conserved molecular patterns that are present in pathogens through a number of pattern recognition receptors. Toll-like receptors (TLRs) are among the well-characterized pattern recognition receptors. At least 10 TLRs have been identified in humans, and one of them, TLR9, is the receptor for bacterial and synthetic DNA containing unmethylated CpG motifs (4). TLR9 is expressed predominantly in B cells and plasmacytoid dendritic cells (pDCs) in humans. Activation of these two cell types by synthetic oligonucleotides containing unmethylated CpG motifs via TLR9 results in a Th1-type immune response which includes the secretion of interferon alpha (IFN- α), IFN- γ , interleukin-12 (IL-12), tumor necrosis factor alpha (TNF- α), and IL-6 with an increase in the levels of costimulatory surface molecules (1, 11, 12, 17, 19, 28). A number of TLR9 agonists are currently being evaluated in clinical trials as therapies for various diseases, including cancers, infectious diseases, allergy, and asthma, and as vaccine adjuvants (1, 10).

The immune response profiles induced via TLR9 stimulation depend on the stimulatory motif and secondary structure present in the oligonucleotides (1–3, 5, 13, 15, 22, 23). Agonists of TLR9 that induce high levels of IFN- α contain either poly(dG)-based hyperstructure-forming sequences (14, 20) or regions of duplex-forming sequences (3, 15, 22). However, TLR9 agonists containing poly(dG) sequences lack pharma-

ceutical properties and have not been evaluated in clinical trials. Agonists of TLR9 containing duplex-forming sequences induce IFN- α production in cell-based assays and are being examined as candidates for the treatment of chronic hepatitis C virus infection in humans.

The type of duplex structure, intra- versus intermolecular, required for IFN- α induction in vitro and in vivo has not been investigated. In the present study, we have selected three sequences that induce high levels of IFN- α in vitro (7, 15, 22) and evaluated their duplex structures and ability to induce TLR9-mediated immune responses in vitro and in vivo in mice and nonhuman primates.

MATERIALS AND METHODS

Synthesis and purification of agonists of TLR9. Agonists of TLR9 and control oligonucleotides were synthesized on a 1- to 2- μ mol scale by using β -cyanoethylphosphoramidite chemistry on a PerSeptive BioSystems 8909 Expedite DNA synthesizer as described earlier (9, 25). The purity of agonists ranged from 90 to 95%, with the rest being shorter by one or two nucleotides ($n - 1$ and $n - 2$) as determined by capillary gel electrophoresis and/or denaturing polyacrylamide gel electrophoresis. All of the compounds contained less than 0.5 EU/ml endotoxin, as determined by the *Limulus* assay (Bio-Whittaker).

Thermal melting study. Agonists of TLR9 at a 2 μ M concentration in 1 ml of 10 mM sodium phosphate buffer, pH 7.2, containing 100 mM NaCl were heated for 5 min at 95°C and allowed to return to room temperature slowly. The solutions were stored at 4°C overnight before the thermal melting temperature (T_m) was measured. Thermal melting experiments were carried out with a Perkin-Elmer Lambda 20 UV/VIS spectrophotometer equipped with a Peltier temperature controller and a multicell holder. Data were collected at each degree by heating or cooling the samples at a rate of 0.5°C/min. The data were collected and analyzed by using Temlab software on a personal computer attached to the instrument. Each experiment was carried out at least two times.

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TABLE 1. Phosphorothioate oligonucleotide sequences and chemical modifications of TLR9 agonists

Compound	Nucleotide sequence ^a	Length	T_m (°C) ^b	
			Dissociation	Association
Agonist 1	5'-TCGTCGTTTTTCGGCGCGCGCCG-3'	22-mer	58 and 82	56
Agonist 2	5'-TCGTCGAACGTTTCGAGATGAT-3'	21-mer	30.1	31.4
Agonist 3	5'-TCRAACRTTCR-X-RCITRCAARCT-5'	22-mer	20.8	21.5
Control 4	5'-ACACACCAACT-X-TCAACCACACA-5'	22-mer	UD	UD

^a All sequences contain a phosphorothioate backbone. R and X represent 2'-deoxy-7-deaza-deoxyguanosine and glycerol. Palindromic sequences in each compound are underlined.

^b Thermal melting experiments were carried out as described in Materials and Methods. Each T_m value is an average of at least two independent experiments, and the values are within $\pm 0.5^\circ\text{C}$. UD stands for undetectable. Dissociation and association indicate values determined from dissociation (heating) and association (cooling) curves, respectively.

Stimulation of HEK293 cells expressing TLR9. HEK293 cells stably expressing mouse TLR9 (Invivogen, San Diego, CA) were cultured in 48-well plates in 250 μl /well Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum in a 5% CO_2 incubator. At 80% confluence, cultures were transiently transfected with 400 ng/ml SEAP (secreted form of human embryonic alkaline phosphatase) reporter plasmid (pNifty2-SEAP; Invivogen) in the presence of 4 μl /ml Lipofectamine (Invitrogen, Carlsbad, CA) in culture medium. Plasmid DNA and Lipofectamine were diluted separately in serum-free medium and incubated at room temperature for 5 min. After incubation, the diluted DNA and Lipofectamine were mixed and the mixtures were incubated at room temperature for 20 min. Aliquots of 25 μl of the DNA-Lipofectamine mixture containing 100 ng of plasmid DNA and 1 μl of Lipofectamine were added to each well of the cell culture plate, and the cultures were continued for 6 h. After transfection, the medium was replaced with fresh culture medium, agonists or control oligonucleotide were added to the cultures, and the cultures were continued for 18 h. At the end of the experiment, 20 μl of culture supernatant was taken from each treatment and used for SEAP assay in accordance with the manufacturer's (Invivogen) protocol. Briefly, culture supernatants were incubated with QUANTI-Blue substrate and the blue color generated was measured by a plate reader at 650 nm. The data are shown as n -fold increases in NF- κB activity over the phosphate-buffered saline (PBS) control.

Isolation of human B cells and pDCs. Peripheral blood mononuclear cells (PBMCs) from blood freshly drawn from healthy volunteers (Research Blood Components, Brighton, MA) were isolated by Ficoll density gradient centrifugation (Histopaque-1077; Sigma). B cells and pDCs were isolated from PBMCs by positive selection with the CD19 and BDCA4 cell isolation kits, respectively, according to the manufacturer's (Miltenyi Biotec) instructions.

Human B-cell proliferation assay. About 1×10^5 B cells/well were stimulated with different concentrations of TLR9 agonists for 66 h and then pulsed with 0.75 μCi of [^3H]thymidine and harvested 8 h later. The incorporation of [^3H]thymidine was measured by scintillation counter, and the data are shown as counts per minute.

Human PBMC and pDC cultures. Human PBMCs and pDCs were plated in 96-well plates at 5×10^6 and 1×10^6 cells/ml, respectively. The compounds dissolved in Dulbecco's PBS were added at a final concentration of 10 $\mu\text{g}/\text{ml}$ to the cell cultures. The cells were then incubated at 37°C for 24 h, and the supernatants were collected for Luminex multiplex assays.

Luminex multiplex assay. The levels of cytokines and chemokines in cell culture supernatants were determined by human cytokine multiplex 25-bead assay (Invitrogen, Camarillo, CA). Data were collected on a Luminex 100 instrument, and fluorescence intensity was transformed into cytokine concentration with StarStation software (Applied Cytometry Systems).

Assessment of mouse serum cytokine levels. Female C57BL/6 mice, 5 to 8 weeks old, were obtained from Charles River Laboratories and maintained in accordance with Idera Pharmaceutical's IACUC-approved animal protocols. Each agonist or control oligonucleotide was administered to mice ($n = 3$) subcutaneously (s.c.) at 1 mg/kg (single dose). Blood was collected by retro-orbital bleeding 2 h after agonist administration, and serum IL-12 levels were determined by sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (9). All reagents, including cytokine antibodies and standards, were purchased from PharMingen (San Diego, CA).

Nonhuman primate study. Healthy young cynomolgus monkeys (*Macaca fascicularis*) weighing approximately 2 to 5 kg were used in this study. All studies were approved by the WIL Research Laboratories or MPI Research Animal Care and Use Committee and were conducted at WIL Research Laboratories, Inc., Ashland, OH, or MPI Research, Inc., Mattawan, MI, respectively. Animals

were monitored daily by veterinarians. Each TLR9 agonist was administered s.c. to three animals on day 1 at 1 mg/kg. Blood samples (1 ml) were collected for plasma cytokine measurement at 0, 1, 2, 4, 8, 24, 48, and 72 h after agonist administration. All animals remained in good health throughout the experiment.

Cytokine analysis of monkey blood samples. Plasma samples were thawed on ice and tested in duplicate for IFN- α , IL-6, and IP-10 with commercial kits obtained from PBL (human IFN- α), BD PharMingen (human IL-6), and R&D Systems (human IP-10) according to the manufacturer's instructions.

Statistical analysis. The results reported for human cell culture assays represent means plus standard deviations obtained from multiple determinations in three or more separate experiments, and the significance of changes was evaluated with Student's t test. For nonhuman primate studies, in any one experiment, there were at least three or four animals in each treatment group. The area under the curve was computed with the MedCalc software. Statistical analyses were carried out by analysis of variance, and the significance of changes was evaluated by the Student-Newman-Keuls post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

TLR9 agonists. The nucleotide sequences of agonists 1 to 3 were taken from published papers (7, 15, 22) and are shown in Table 1. Agonists 1 and 2 had natural CpG stimulatory motifs at the 5' end and a duplex-forming sequence toward the 3' end. Agonist 3 contained two short oligomers linked through their 3' ends (7). Agonist 3 also contained synthetic CpR (R = 2'-deoxy-7-deazaguanosine) motifs in a palindromic sequence that allows the formation of a duplex structure. All three agonists were of similar length (21 or 22 nucleotides) and were characterized by mass spectral analysis for sequence integrity and by capillary and/or slab gel electrophoresis for purity.

Thermal melting study. The three agonists were studied for secondary structure formation by UV thermal melting experiments. At a concentration of 2 μM , all three agonists showed cooperative dissociation curves as the temperature increased from 5 to 95°C (Fig. 1), and the T_m s calculated are shown in Table 1. Agonists 2 and 3 showed monophasic melting transitions with T_m s of 30.1 and 20.8°C , respectively. On the contrary, agonist 1 showed a biphasic melting transition with T_m s of 58 and 82°C (Fig. 1A). Both agonists 2 and 3 had association curves similar to their respective dissociation curves (Fig. 1B and C), with T_m s of 31.4 and 21.5°C , respectively. The association curve of agonist 1 was monophasic, with a T_m of 56°C , unlike its biphasic dissociation curve with lower hyperchromicity (Fig. 1A). These results suggest that agonists 2 and 3 form a single homogeneous structure, while agonist 1 forms at least two different structural populations under the experimental conditions used. Possible secondary structures formed by each

FIG. 1. UV thermal melting curves of agonists 1 (A), 2 (B), and 3 (C). The thick line in each plot represents the dissociation curve obtained as the temperature increased from 5 to 95 $^{\circ}\text{C}$ (heating curve), and the thin line represents the association curve obtained during cooling of the samples from 95 to 5 $^{\circ}\text{C}$ (cooling curve). The data shown are representative of at least two independent experiments. See Table 1 for sequence information.

agonist are shown in Fig. 1 along with the theoretically calculated ΔG values.

Activation of TLR9 by agonists. To determine if the agonists were recognized by TLR9, we used HEK293 cells expressing mouse TLR9. All three agonists activated NF- κ B, unlike the control compound lacking a stimulatory CpG motif, indicating that TLR9 recognizes CpG and CpR dinucleotides in the agonists (Fig. 2).

Activation of human PBMCs and pDCs and induction of IFN- α secretion. Agonists were evaluated for their biological effects on freshly isolated human PBMCs. All three agonists induced the production of IFN- α and other cytokines and chemokines, including IL-12, IP-10, IL-6, IL-2R, MIP-1 α , and MIP-1 β , by human PBMCs (Fig. 3). Of the three compounds, agonist 3 induced the highest levels of IFN- α in PBMC cultures. All three agonists were also tested for the ability to induce cytokine and chemokine production by freshly isolated human pDCs, which express TLR9 (Fig. 4). All three agonists induced the production of similar levels of IL-6, IL-12, IFN- α , IP-10, IL-2R, MCP-1, MIP-1 α , and MIP-1 β in pDC cultures (Fig. 4). All three agonists produced significantly higher levels of cytokines and chemokines compared with the control oligonucleotide in both human PBMCs and pDCs. The statistical

FIG. 2. Activation of HEK293 cells expressing mouse TLR9 by agonists at a 10 $\mu\text{g}/\text{ml}$ concentration. The data shown are representative of three or more independent experiments.

significance of differences between the compounds is indicated in Fig. 3 and 4.

Activation of human B cells by agonists. As B cells also express TLR9, we further evaluated the abilities of the agonists to induce B-cell proliferation. All three agonists produced a dose-dependent increase in the proliferation of B cells, and the extents of proliferation were similar among the agonists (Fig. 5). The control compound did not induce B-cell proliferation, suggesting that the proliferation was sequence specific (Fig. 5).

IL-12 induction in vivo in mice. Administration of 1 mg/kg agonist 3 or 2 s.c. to mice resulted in the elevation of serum IL-12 levels to about 115 and 17.5 ng/ml, respectively (Fig. 6). On the contrary, administration of agonist 1 at the same dose to mice did not result in any change in the levels of serum IL-12 compared with the control compound (Fig. 6).

Cytokine profiles of TLR9 agonists in nonhuman primates.

Each agonist was administered s.c. to cynomolgus monkeys at a single dose of 1 mg/kg, blood was collected at different time intervals, and plasma levels of IFN- α , IP-10, and IL-6 were determined by ELISA (Fig. 7). Administration of agonist 2 or 3 to monkeys resulted in maximal levels of IFN- α induction by 8 h (Fig. 7A). Agonists 2 and 3 induced similar levels of IFN- α . A sustained level of IFN- α was observed up to 72 h postadministration in the plasma of monkeys that received agonist 3. In contrast, plasma IFN- α of monkeys that received agonist 2 returned to predose levels by 48 h (Fig. 7A). Agonist 1 induced lower levels of IFN- α than did agonists 2 and 3; the maximal levels were observed at around 24 h, and the levels returned to the background by 48 h (Fig. 7A). Agonists 1 and 2 produced similar levels of IP-10, with peak concentrations reached by 4 to 8 h after agonist administration (Fig. 7B). Agonist 3 induced levels of IP-10 more than two times higher than those induced by agonists 1 and 2. Both agonists 2 and 3 induced minimal levels (<50 pg/ml) of IL-6 (Fig. 7C). On the contrary, agonist 1 induced relatively higher levels of IL-6; a maximal concentration of about 250 pg/ml was measured at 8 h after agonist administration (Fig. 7C).

FIG. 3. Induction of cytokine and chemokine production by TLR9 agonists in human PBMC cultures. PBMCs isolated from fresh blood obtained from healthy human volunteers were cultured in the presence or absence of 10 μ g/ml TLR9 agonists for 24 h as described in Materials and Methods. Supernatants were collected and analyzed by Luminex multiplex assay. The data shown are representative of three or more independent experiments. M and C stand for medium and control oligonucleotide 4, respectively. The statistical significance of differences between the agonists was determined and is indicated by the symbols #, \$, and *, where $P < 0.05$ for 1 versus 2, 2 versus 3, and 1 versus 3, respectively.

DISCUSSION

TLR9 agonists promote B-cell proliferation, immunoglobulin production, and the secretion of a number of Th1-type cytokines, including IL-12, IFN- γ , IL-6, and IFN- α . TLR9 recognizes synthetic and bacterial DNA that contains unmethylated CpG motifs and initiates an immune signaling cascade in a MyD88-dependent fashion (10, 22), leading to the activation of the transcription factors NF- κ B (18) and AP-1 (24). While a CpG dinucleotide is essential for TLR9 activation, a number of other factors, such as the nucleotides flanking the CpG dinucleotide, the presence of an accessible 5' end, the position of the CpG dinucleotide in oligonucleotides, and the secondary structures of the oligonucleotides, play critical roles in the activation of immune cells (1).

Our previous studies with mouse and human cell-based assays showed that CpG oligonucleotides containing a hairpin structure at the 3', but not the 5', end were immunostimulatory (2, 5). These results were consistent with our studies showing that an accessible 5' end of a CpG oligonucleotide is required for TLR9 stimulation (6–9, 26, 27). In fact, the 3' hairpin structure-forming CpG oligonucleotides induce high levels of IFN- α production by human pDCs, suggesting that a secondary structure is required for IFN- α induction by TLR9 agonists (2, 3, 5). However, surprisingly, such intramolecular hairpin

structure-forming CpG oligonucleotides did not induce immune responses in vivo (our unpublished results).

CpG oligonucleotides containing palindromic sequences, referred to as class C, activate B cells and pDCs and induce the production of high levels of IFN- α in vitro (3, 15, 22). Because of their ability to induce IFN- α production, some of these compounds have been evaluated as candidates in clinical trials with hepatitis C-infected patients (16, 21). The presence of longer palindromic sequences in oligonucleotides can facilitate the formation of both intra- and intermolecular duplexes. It is not known whether different types of TLR9 agonists that induce high levels of IFN- α form intra- or intermolecular duplexes or if their secondary structures affect the induction of immune responses in vivo. In the present study, we evaluated three different TLR9 agonists that have three distinct sequence compositions (7, 15, 22) but have a common feature of forming duplexes and inducing the production of high levels of IFN- α in cell-based assays. Additionally, agonists 1 to 3 have seven, four, and six CpG (R) dinucleotides, respectively. However, studies have shown that the presence of more than three CpG dinucleotides in a 22- to 25-mer oligonucleotide does not enhance activity further (12). The goal of this study was to determine the type of structures formed and the immune response profiles produced by these compounds in vitro and in vivo.

FIG. 4. Induction of cytokine and chemokine production by TLR9 agonists in human pDC cultures. pDCs isolated from PBMCs of healthy human volunteers were cultured in the presence or absence of 10 μ g/ml TLR9 agonists for 24 h as described in Materials and Methods. Supernatants were collected and analyzed by Luminex multiplex assay. The data shown are representative of three or more independent experiments. M and C stand for medium and control oligonucleotide 4, respectively. The statistical significance of differences between the agonists was determined and is indicated by the symbols #, \$, and *, where $P < 0.05$ for 1 versus 2, 2 versus 3, and 1 versus 3, respectively.

FIG. 5. Human B-cell proliferation induced by agonists at various concentrations of agonists 1 ($\diamond$), 2 ($\square$), and 3 ($\triangle$); control oligonucleotide 4 ($\bullet$); and PBS ($\blacktriangle$). Experiments were carried out as described in Materials and Methods. The data shown are representative of three or more independent experiments.

In UV thermal melting studies, agonists 2 and 3 showed a monophasic thermal melting transition, suggesting that they formed a single type of duplex structure. In fact, the association curve was very similar to the dissociation curve. In contrast, agonist 1 showed a biphasic thermal melting curve with two distinct transitions and the association curve showed a monophasic transition with lower hyperchromicity. These results suggest that agonist 1 forms two distinct populations of secondary structures at equilibrium and upon rapid cooling it forms a kinetically more feasible secondary structure with lower hyperchromicity. The type of 3'-3'-attached structure in agonist 3 does not permit the formation of a hairpin structure between the two branches. Similarly, a short, 10-nucleotide-long palindromic sequence in each branch of agonist 3 does not permit the formation of the intramolecular hairpin type of structure. Therefore, agonist 3 forms only an intermolecular duplex. In contrast, agonist 1 forms a mixture of both intra- and intermolecular duplexes. Based on the thermal melting study results, agonist 2 appears to form only an intermolecular duplex.

In HEK293 and primary human cell-based assays, all three compounds activated TLR9 and produced TLR9-mediated immune responses. In fact, all three compounds induced comparable levels of IFN- α and other cytokines and chemokines in human pDCs and induced similar levels of human B-cell proliferation. These results are consistent with our earlier studies showing that both inter- and intramolecular duplex-forming CpG oligonucleotides induce immune responses in vitro (2, 5). The differences in the activities observed in vitro could be a result of differences in oligonucleotide sequence composition

FIG. 6. In vivo IL-12 induction by agonists in mice at a 1 mg/kg dose. Blood was collected 2 h after agonist administration, and serum IL-12 was determined by ELISA as described in Materials and Methods. The data shown are representative of three independent experiments.

FIG. 7. In vivo IFN- α (A), IP-10 (B), and IL-6 (C) induction by agonists 1 ($\diamond$), 2 ($\square$), and 3 ($\triangle$) in cynomolgus monkeys. The plasma levels of IFN- α produced by agonist 3 are statistically significantly different from those produced by agonists 1 and 2.

rather than the number of CpG dinucleotides present in each agonist.

In vivo in mice, agonists 2 and 3, which form intermolecular duplexes, induced IL-12 production. Agonist 1, which can form both intra- and intermolecular duplexes, failed to induce IL-12 production. These results are consistent with other intramolecular hairpin-forming oligonucleotides that were studied in our laboratory and found not to induce immune responses in vivo in mice (our unpublished results).

Not many studies of TLR9 agonists in vivo in monkeys have been reported. Comparison of the three agonists in nonhuman primates showed that agonist 1 induced lower levels of IFN- α and IP-10 than did agonists 2 and 3. Agonist 3, which forms a multimeric intermolecular duplex, induced significantly higher and sustained levels of IFN- α up to 72 h after administration at the dose level tested. Additionally, the immune response profiles induced by agonists 2 and 3 differed as agonist 1 induced IL-6 in nonhuman primates whereas agonists 2 and 3 did not.

In summary, the present in vitro and in vivo studies suggest that CpG oligonucleotides containing palindromic sequences can form both intra- and intermolecular duplexes. In vitro studies show that both intra- and intermolecular secondary structure-forming CpG oligonucleotides induce potent cytokine production, including IFN- α induction in pDCs. Intramolecular secondary structure-forming CpG oligonucleotides are less potent than intermolecular secondary structure-forming CpG oligonucleotides in vivo, however. Intermolecular secondary structure-forming agonists of TLR9 induce potent immune responses in vivo, including IFN- α induction in nonhu-

man primates. Based on these results, we have selected agonist 3, which forms an intermolecular duplex and induces high levels of IFN- α in nonhuman primates, as a candidate for the treatment of hepatitis C. Agonist 3 is currently being evaluated in a phase I clinical trial with hepatitis C-infected patients.

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