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Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenicity of a peptide malaria vaccine in *Aotus* monkeys

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Abstract

Synthetic peptide and recombinant protein vaccines are optimally immunogenic when delivered with an effective adjuvant. Candidate vaccines currently insufficiently immunogenic may induce a protective immunity if they could be delivered with more effective adjuvants. For example, immunogens that induce promising responses when administered to mice with complete and incomplete Freund's adjuvants perform less well in primate animal models where complete Freund's adjuvant is not used. We report the use of synthetic oligodeoxynucleotides containing CpG motifs, the sequences of which are based on immunostimulatory bacterial DNA sequences, to enhance the immune response in *Aotus* monkeys to a synthetic peptide malaria vaccine. Monkeys were immunized with the synthetic peptide PADRE 45, a synthetic peptide containing amino acid sequences derived from the circumsporozoite protein (CSP) from *Plasmodium falciparum*, and delivered in an emulsion of saline and Montanide 720, a mannide oleate in oil solution, that also contained one of three oligodeoxynucleotides. The animals receiving oligodeoxynucleotides containing either three or four CpG motifs produced antibodies that bound a recombinant CSP as measured in ELISA, and reacted with *P. falciparum* sporozoites in a sporozoite immunofluorescent test. These responses were significantly greater than those seen in animals receiving the oligodeoxynucleotide without CpG motifs. These data indicate that oligodeoxynucleotides containing CpG motifs improve immunogenicity of peptide immunogens in non-human primates, and may be immunopotentiators useful in humans.

Keywords: CpG motif; Oligodeoxynucleotide; Immunization; Malaria; *Aotus*

1. Introduction

Synthetic peptide and recombinant protein vaccines are usually poorly immunogenic when administered alone. Very large increases in immunogenicity can be obtained by delivering these vaccines with powerful adjuvants. Promising malaria vaccines, such as RTS,S [1] and formulations containing Erythrocyte Binding Antigen-175 (EBA-175) [2], may be significantly improved simply by using more immunogenic adjuvants. In Merozoite Surface Antigen-1 (MSP-1) vaccine studies performed in *Aotus* monkeys, only formulations containing complete Freund's adjuvant have induced protection [3,4]. Use of this adjuvant in

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primates, however, is now strongly discouraged or simply prohibited because of its significant side effects, and it cannot be considered for use in humans for the same reason. The need for safe but potent adjuvants and immunopotentiators is greater than ever, particularly in primate animal models.

The vertebrate immune system can be activated by a variety of microbial components. Among these components are CpG dinucleotides in particular base contexts found in bacterial DNA [5,6]. CpG dinucleotides are present in the predicted frequency of 1 in 16 in bacteria and virus genomes, but are seen only 25% as frequently in mammalian DNA, and, unlike microbial CpG, the mammalian CpG are highly methylated [7]. Thus, the vertebrate innate immune system may have evolved the ability to detect bacterial infection based on recognition of unmethylated CpG motifs in the bacterial DNA. Most viruses have managed to evade the immune system by eliminating stimulatory CpG motifs from their genomes. Furthermore, certain adenoviral genomes contain "neutralizing" CpG motifs that can actually inhibit immune activation by otherwise immunostimulatory CpG motifs, such as those commonly found in bacterial genomes [8].

Once recognized, CpG motifs activate a wide variety of innate immune responses. Bacterial DNA or single-stranded synthetic oligodeoxynucleotides (ODN) containing these immunostimulatory CpG motifs activate B cells to secrete IL-6 and IL-10, immunoglobulin, and to express increased levels of costimulatory molecules [5,6,9–12]. Monocytes and macrophages are activated to secrete IL-12, leading to NK activation and IFN-γ secretion [13–15]. Moreover, B cells, monocytes, and dendritic cells are all activated by CpG ODN to have increased expression of costimulatory molecules, leading to enhanced antigen presentation [5,6,16,17]. These data suggest the possibility that CpG ODN could have an adjuvant effect inducing the antigen-specific generation of B and T cell immune responses. Indeed, several studies have recently shown that CpG ODNs are potent adjuvants for inducing Th1 immune responses to a wide variety of antigens [6,18–21]. In addition, CpG motifs within DNA vaccines appear to provide essential adjuvant activity since immune responses are abrogated in mice if potent motifs are removed or methylated [22–24]. However, all of these studies were performed in mice, and mice are often more responsive to immune stimulants than are primates. One additional factor that may limit the applicability of these mouse data to humans is the recent finding that CpG motifs are species-specific, with the flanking bases and spacing between adjacent motifs determining whether a given CpG dinucleotide is stimulatory for a given species (Krieg and Davis, unpublished results). While murine immune cells respond to a wide variety of CpG motifs, cells obtained from humans and other primates respond to a much more restricted subset.

The *Aotus* monkey is a valuable model to evaluate malaria vaccines because of its susceptibility to infection with both *Plasmodium falciparum* and *P. vivax* [25]. We have already shown the possibility of using CpG-containing bacterial DNA to augment antibody responses with a DNA vaccine against hepatitis B in *Aotus* monkeys [26]. In that case, *E. coli* DNA was used as a general source of CpG motifs since we had not yet identified specific effective CpG motifs for use in a non-human primate such as the *Aotus* monkey. In any event, it is not possible to use CpG-containing synthetic ODNs with plasmid DNA owing to interference of plasmid uptake into cells [27]. Apparently, the synthetic nuclease resistant phosphorothioate backbone of the ODN that is essential for in vivo use outcompetes the natural phosphodiester backbone of the plasmid for DNA-binding sites on the cell membrane. Thus, it is necessary to clone CpG motifs into the vector backbone, or to use a protein antigen with an effective CpG ODN.

We have now used an in vitro assay to identify CpG motifs that are stimulatory for *Aotus* monkey peripheral blood mononuclear cells (PBMC) and have evaluated the efficacy of these CpG ODNs to augment in vivo malaria-specific responses against a peptide containing amino acid sequences derived from the circumsporozoite protein (CSP) from *P. falciparum*.

2. Materials and methods

Based on preliminary in vitro testing, three ODNs were selected for in vivo use with the peptide vaccine: 1968, TCGTCGCTGTTGTCGTTTCTT; 2006, TCGTCGTTTTGTCGTTTTGTCGTT; and 2041, CTGGTCTTTCTGGTTTTTTCTGG. ODN 2041, lacking CpG dinucleotides, serves as the negative control. All ODNs were synthesized under GMP, and with a nuclease-resistant phosphorothioate backbone, by Hybridon Specialty Products (Milford, MA). Endotoxin levels in the ODNs were undetectable (< 1.0 EU/mg). The Na^+ salts of the ODN were ethanol precipitated and then resuspended in 10 mM Tris (pH 7.0) 1 mM EDTA for storage at −20°C before dilution into saline for injection.

2.1. Peptide

PADRE 45 is a synthetic peptide containing amino acid sequences derived from the circumsporozoite protein (CSP) from *P. falciparum*. It was synthesized by F-moc stepwise phase peptide synthesis using an automatic PE/ABI synthesizer with conductivity monitoring of F-moc deprotection. After synthesis, the peptide

was deprotected and cleaved from the resin. After cleavage and lyophilization, PADRE 45 peptide was purified on Reverse Phase HPLC using linear gradient of water/0.1% TFA and acetonitrile/0.1% TFA. The sequence of PADRE 45 is as follows: aKXVAAWTLKAa(PNANPNANPNVDPNANPNA-NPNVDPNANPN)-GGS where X = l-cyclohexylalanine, and where upper case letters indicate L-amino acids and lower case letters indicate D-amino acids.

2.2. Monkeys

Nine Panamanian adult (male and female) *Aotus lemurinus lemurinus* (karyotype VIII or IX) monkeys [28] were maintained in the animal facility of the Gorgas Memorial Laboratory in Panama City, Republic of Panama. *Aotus l. lemurinus* monkeys were obtained in western Panama. Upon arrival at the laboratory, each animal was given a physical examination, weighed and sexed, identified by a metal neck tag with an accession number, administered thiabendazole orally for treatment of endoparasites (100 mg base/kg), and vaccinated against *Herpes simplex*, *Herpes tamarinus* (New England Regional Primate Research Center, Southborough, MA, USA) and *Klebsiella pneumoniae* [29]. The animals were housed and cared for as previously described [29]. About 1 month after arrival, each monkey was tattooed with an identification number and a thick blood film examined to exclude naturally occurring plasmodial infections. The animals remained in quarantine for a minimum of 90 days before being transferred to areas devoted to housing monkeys for malaria studies. The weight of the monkeys when inoculated ranged from 700–800 g. All monkeys used in these experiments were wild caught adult monkeys that had been previously infected one time with *P. falciparum* and one time with *P. vivax* by the intravenous inoculation of infected erythrocytes. All animals received follow-up drug cures. None had been exposed to *P. falciparum* sporozoites.

2.3. Immunizations

The nine monkeys were randomly assigned to one of three groups, each group containing three monkeys. The animals received three immunizations at 3-week intervals. The vaccine formulations for the three groups were identical except for substitution of a different ODN. Each animal received 100 μg of PADRE 45 and 500 μg of ODN in an emulsion of 1/3 saline, 2/3 lipid adjuvant. The lipid adjuvant used was Montanide 720 (Seppic SA, Paris), a solution of mannide oleate in oil. An emulsion was prepared by repeatedly forcing the reagents through a small bore tube connecting two syringes. Each monkey received a single 400 μl injection in the quadriceps.

2.4. ELISA

The ELISA was performed as previously described [30], with minor modification. The target antigen was a *P. falciparum* recombinant circumsporozoite protein (rPfCSP), produced in *E. coli*, and based on the sequence of the 3D7 clone of the NF 54 strain (T.P. Le and S.L. Hoffman, manuscript submitted). The rPfCSP consists of residues 94 to 397, and contains 36 copies of the NANP repeat and four copies of the NVDP repeat. Briefly, 50 μl of 0.25 μg/ml of the rPfCSP in PBS was added into wells of the Immulon II ELISA plates (Dynatech Laboratory Inc., Chantilly, VA) and incubated for 6 h at room temperature. The wells were washed 3 times with PBS containing 0.05% Tween 20 (washing buffer) and incubated overnight at 4°C with 100 μl of 5% nonfat dry milk in PBS (blocking buffer). After washing 3 times with washing buffer, the wells were incubated for 2 h with 50 μl of two-fold dilutions of pre-and post immunized monkey sera diluted in PBS containing 3% nonfat dry milk (diluting buffer). The wells were washed three times, incubated for 1 h with peroxidase labeled goat anti-human IgG (H + L) (Kirkegaard & Perry, Gaithersburg, MD) diluted 1:4000 in diluting buffer, then washed again three times. The wells were incubated for 20 min with 100 μl of a solution containing ABTS substrate (2,2′ azino-di-3-ethylbenzthiazoline sulfonate) (Kirkegaard & Perry, Gaithersburg, MD) and H_2O_2. Color reaction was measured in a micro-ELISA automated reader (Dynatech, MR5000) at OD 410 nm. All reaction steps except blocking were performed at room temperature. The mean ± SD of the readings of the quadruplicate assays was recorded.

2.5. Immunofluorescent antibody test (IFAT)

The IFAT was performed as previously described [31], with a minor modifications. Briefly, 20 μl of two fold-dilutions of pre- and post-immunized monkey sera diluted in PBS were added to wells of air-dried *P. falciparum* sporozoites (NF 54 strain) and the slides were incubated for 30 min at 37°C. The wells were washed three times with PBS and further incubated for 30 min with 20 μl of 1:50 dilution of fluorescein-labeled goat anti-human IgG (H + L) (Kirkegaard & Perry, Gaithersburg, MD). The wells were washed three times with PBS, mounted in 10% glycerol in PBS and examined under an Olympus UV microscope. The positive control was sera from volunteers immunized with radiation attenuated *P. falciparum* sporozoites.

Table 1
Induction of B cell proliferation by species specific CpG motifs. nd = not done; Cells used were whole spleen (mouse) or whole PBMC (all others). The results are reported as means of stimulation indexes for the indicated number of individuals compared to cells cultured in medium alone for [^{3}H] thymidine incorporation performed as previously described [3]. The proliferating cells were identified as B cells using flow cytometry (not shown). The stimulation indexes cannot be compared directly across species because the experiments with different species were performed on different days

	Mouse (Balb/c)	*Aotus* ($N=4$)	*M. cynomolgus* ($N=4$)	Chimpanzee ($N=5$)	Human ($N=4$)
1826	143	nd	4.9	0.8	2.0
1968	nd	1.5	3.5	61.6	82
2006	145	2.6	7.7	99.7	141

2.6. Statistics

Statistical tests were performed in SPSS for Windows, 8.0.

3. Results

3.1. Selection of CpG ODN for immunization of Aotus monkeys

In previous studies, we found that the immune stimulatory effects of ODN containing CpG dinucleotides depended entirely on the bases flanking the CpGs, the number of CpGs in an oligo, and the spacing between the individual CpG motifs [5,8–11]. Depending on the exact motif, the adjuvant effect of CpG ODNs can vary enormously [8,32]. However, we have observed that the relative adjuvant effects of different CpG in mice correlate with the level of induction of B cell proliferation (Davis and Krieg, unpublished observation). To identify ODNs for in vivo immunization of *Aotus* monkeys, preliminary in vitro experiments were performed in which a panel of ODN-bearing different CpG motifs were compared for their ability to induce proliferation of *Aotus* B cells, as well as those of humans and other species. This may be useful in predicting which ODNs may work best in humans. Interestingly, CpG ODN 1826, which we had previously reported to be an extremely potent B cell mitogen and adjuvant for immunization of mice [5], was mitogenic for a lower primate, *Macaca cynomolgus*, but not for higher primates such as chimpanzees or humans (Table 1). We therefore screened several hundred ODNs bearing a wide range of CpG motifs for their mitogenic effects on human B cells, and identified several that were especially mitogenic (A.M. Krieg et al., manuscript in preparation). Two of the most potent ODNs, 1968 and 2006, were also found to be mitogenic for *Aotus* B cells (Table 1). Therefore, both of these ODNs were tested in vivo for their adjuvant activities, with a non-CpG ODN, 2041, included as a negative control.

3.2. Use of CpG ODN to augment humoral responses against malaria peptide

3.2.1. ELISA

Serum samples collected at two time-points after two immunizations (days 30 and 41) and samples collected at two time points after three immunizations (days 56 and 70) were analyzed. The OD values for serial dilutions of each serum sample obtained in ELISA were used to calculate through interpolation the estimated serum dilution of a given serum sample required to yield an OD of 0.5. Briefly, titer-OD x,y pairs bracketing an OD of 0.5 were $\log_{10}$-transformed, the slope and y intercept calculated, then the $\log_{10}$ titer at OD = 0.5 determined. The antilog$_{10}$ of that value was then calculated. These values are displayed in Fig. 1. There was substantial variance within groups so the data were transformed ($\log_{10}$), and serum titers at 0.5

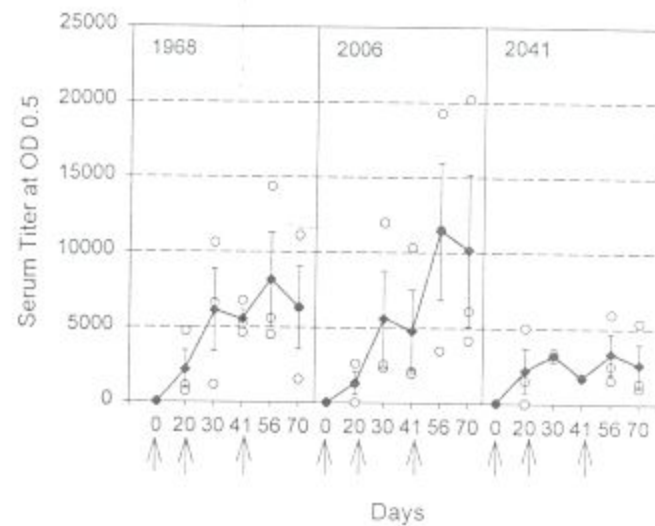

Fig. 1. The serum antibody titers yielding an estimated OD of 0.5 are plotted against the day the animals were bled. The monkeys were immunized with a synthetic peptide containing amino acid sequences derived from the circumsporozoite protein (CSP) from *P. falciparum*. Immunizations occurred on days 0, 21 and 41 (arrows). Each panel is labeled with the ODN the animals received. Open circles (○) are individual animals, filled diamonds (◆) indicate the mean OD on that day. Bars represent standard error of the mean.

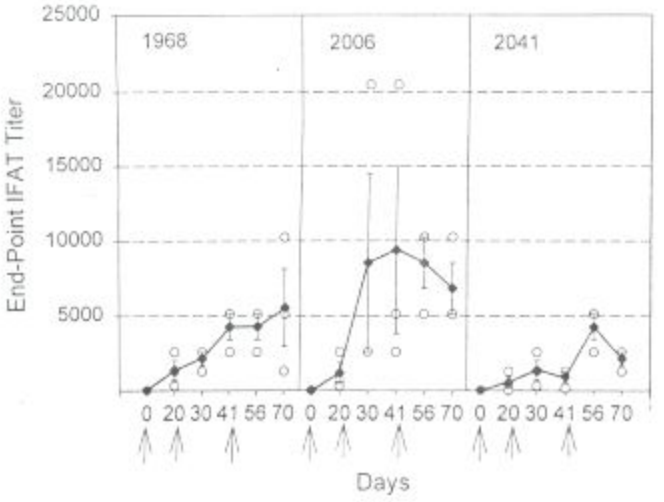

Fig. 2. End-point serum antibody titers in an immunofluorescent antibody test plotted against the day the animals were bled. The monkeys were immunized with a synthetic peptide containing amino acid sequences derived from the circumsporozoite protein (CSP) from *P. falciparum*. The antigen was freeze-dried *P. falciparum* sporozoites. Immunizations occurred on days 0, 21 and 41 (arrows). Each panel is labeled with the ODN the animals received. Open circles (○) are individual animals, filled diamonds (◆) indicate the mean end-point serum titer on that day. Bars represent standard error of the mean.

OD were compared in an independent samples *t*-test. The mean $\log_{10}$-transformed serum titers of the monkeys that received CpG ODN 1968 and CpG ODN 2006 were both significantly greater ($p=0.01$ and $p=0.012$ respectively) that the control nonCpG ODN 2041. The untransformed data were evaluated in a nonparametric test (Mann–Whitney *U*-test, two-tailed). Again, monkeys immunized with either CpG ODN 1968 or CpG ODN 2006 had significantly higher antibody titers than did those immunized with the control ODN 2041 ($p=0.018$ and $p=0.015$, respectively). Differences between animals immunized with CpG ODN 1968 and CpG ODN 2006 were not significant.

3.2.2. Immunofluorescent antibody test (IFAT)

Serum samples collected at two time-points after two immunizations (days 30 and 41) and samples collected at two time points after three immunizations (days 56 and 70) were analyzed. End-point serum titers to *P. falciparum* sporozoites for these serum samples were $\log_{10}$-transformed and differences were evaluated in an independent samples *t*-test. Monkeys immunized with either CpG ODN 1968 or CpG ODN 2006 were significantly different from antibody levels in animals immunized with the control nonCpG ODN 2041 ($p=0.03$, and $p=0.001$, respectively). Monkeys immunized with CpG ODN 2006 also had significantly higher antibody titers than did those immunized with the CpG ODN 1968 ($p=0.034$) (Fig. 2). Mann–Whitney *U*-test (two-tailed) of untransformed values also showed that antibody levels in animals immunized with CpG ODN 1968 and CpG ODN 2006 were significantly different from levels in animals immunized with the control nonCpG ODN 2041 ($p=0.036$ and $p=0.001$, respectively). Monkeys immunized with CpG ODN 2006 also had significantly higher antibody titers than did those immunized with the CpG ODN 1968 ($p=0.05$).

4. Discussion

This is the first report demonstrating that CpG ODNs can be used to enhance the immune response in monkeys immunized with a synthetic peptide vaccine. This is a potentially valuable finding because it suggests that ODNs may be of use in improving the immune response in humans immunized with synthetic peptide or recombinant protein vaccines. Initial studies designed to define the protective potential of candidate vaccine immunogens ("proof of principle") are often done in mice using complete Freund's adjuvant. The inability to use this "gold standard" adjuvant has made the transition from mouse to primate and human work more difficult, because adjuvants substituted for complete Freund's adjuvant in primate studies have not been as effective. The ability of CpG ODNs to potentiate the immunogenicity of synthetic peptides when delivered with a lipid adjuvant (Montanide 720 in this case) may make studies in mice and primates more comparable.

In this study, both CpG ODN 1968 and CpG ODN 2006 induced higher antibody titers, as measured in both ELISA and IFAT, than did the control, nonCpG ODN 2041. In addition, CpG ODN 2006 slightly outperformed ODN 1968 as measured in IFAT. This difference was not seen in ELISA. CpG ODN 2006 contains four CpG sequences while CpG ODN 1968 contains only three, but it is not clear whether the additional CpG in CpG ODN 2006 is actually responsible for its enhanced performance.

Several features may have contributed to the adjuvant effect of the CpG ODN. First, the CpG ODN-induced B cell activation synergizes with signals received through the antigen receptor [5]. Thus, B cells that bind their specific antigen through the antigen receptor would be preferentially activated by CpG DNA, as compared to B cells that do not bind their specific antigen. Second, CpG ODN induces changes in the expression of adhesion molecules by dendritic cells, which may enhance their trafficking to lymph nodes [16]. Third, CpG ODN enhances the expression of class II MHC and costimulatory molecules such as B7-1 and B7-2, which should also improve their ability to induce B and T cell immune responses [6,16].

These data suggest that CpG ODNs may be effective adjuvants for enhancing immune responses to peptide vaccines. Preclinical safety studies indicate that CpG DNA is well tolerated in primates at doses up to 10 mg/kg (A.M. Krieg et al., manuscript in preparation). Advantages of CpG ODNs compared to other adjuvants include a lack of toxicity, the extreme stability of these nuclease-resistant phosphorothioate-modified CpG ODNs against degradation, their ease of formulation, and the low cost of manufacture. We estimate that a human dose will cost less than a dollar at current commercial prices for GMP grade CpG ODN, and possibly only cents per dose once manufacturing is scaled up. Based on the in vitro studies shown in Table 1, it is clear that CpG ODNs which are mitogenic for mouse and lower primate B cells may not necessarily be mitogenic for those of humans or higher primates. Therefore, we suggest screening studies be performed using human cells to identify ODNs that may be optimal for human immunization. Further studies will be required to determine whether CpG ODN will have similar or even better in vivo adjuvant properties in humans.

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