INFECTION AND IMMUNITY, Mar. 2001, p. 1643–1649 Vol. 69, No. 3
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.3.1643–1649.2001

Interleukin-12- and Gamma Interferon-Dependent Protection against Malaria Conferred by CpG Oligodeoxynucleotide in Mice

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Received 6 September 2000/Returned for modification 16 October 2000/Accepted 12 December 2000

Unmethylated CpG dinucleotides in bacterial DNA or synthetic oligodeoxynucleotides (ODNs) cause B-cell proliferation and immunoglobulin secretion, monocyte cytokine secretion, and activation of natural killer (NK) cell lytic activity and gamma interferon (IFN-γ) secretion in vivo and in vitro. The potent Th1-like immune activation by CpG ODNs suggests a possible utility for enhancing innate immunity against infectious pathogens. We therefore investigated whether the innate immune response could protect against malaria. Treatment of mice with CpG ODN 1826 (TCCATGACGTTCCTGACGTT, with the CpG dinucleotides underlined) or 1585 (ggGGTCAACGTTGAggggG, with g representing diester linkages and phosphorothioate linkages being to the right of lowercase letters) in the absence of antigen 1 to 2 days prior to challenge with *Plasmodium yoelii* sporozoites conferred sterile protection against infection. A higher level of protection was consistently induced by CpG ODN 1826 compared with CpG ODN 1585. The protective effects of both CpG ODNs were dependent on interleukin-12, as well as IFN-γ. Moreover, $CD8^+$ T cells (but not $CD4^+$ T cells), NK cells, and nitric oxide were implicated in the CpG ODN 1585-induced protection. These data establish that the protective mechanism induced by administration of CpG ODN 1585 in the absence of parasite antigen is similar in nature to the mechanism induced by immunization with radiation-attenuated *P. yoelii* sporozoites or with plasmid DNA encoding preerythrocytic-stage *P. yoelii* antigens. We were unable to confirm whether $CD8^+$ T cells, NK cells, or nitric oxide were required for the CpG ODN 1826-induced protection, but this may reflect differences in the potency of the ODNs rather than a real difference in the mechanism of action of the two ODNs. This is the first report that stimulation of the innate immune system by CpG immunostimulatory motifs can confer sterile protection against malaria.

It is estimated that the causative agents of malaria, such as *Plasmodium falciparum* or *Plasmodium vivax*, result in an estimated 300 to 500 million new infections and 1.5 to 2.7 million deaths annually (35). In addition, tens of millions of travelers from countries where malaria is not endemic visit countries where it is, and many of these succumb to illness during their travels or after returning home. In the latter case, there is a particular risk of failure to rapidly diagnose and initiate treatment, owing to the lack of experience with the disease of many local physicians.

The treatment and prevention of malaria have traditionally depended upon antimalarial drugs targeted against the parasite. Although historically effective, many of the parasites that cause malaria have now developed resistance to such drugs, and there are few new drug candidates on the horizon (14). Thus, new and more effective methods to prevent and treat this widespread and serious disease are required. Considerable effort has been put into the development of vaccines designed to induce specific antiparasite immune responses. While there has been substantial progress in this endeavor (14, 27), no antimalarial vaccine has yet been licensed.

It is now well established that there are two general systems of immunity against pathogen infection: innate immunity, which uses proteins encoded in the germ line that recognize molecules unique to infectious organisms, and adaptive (acquired) immunity, which uses T and B lymphocytes expressing distinct antigen receptors that recognize pathogen-derived peptides. Innate immunity has been considered only to provide rapid, short-term, incomplete antimicrobial host defense until the slower antigen-specific acquired immune response develops. Recently, however, it has been suggested (1, 10, 28) that the innate immune response may play a pivotal role in immune regulation and the development of host immunity by determining which antigens the acquired immune system responds to and the nature of that response. Almost all efforts towards the development of an effective malaria vaccine, however, have focused on the effector phase of the antigen-specific adaptive immune response (14, 27). In contrast, here we have investigated the role of the innate immune response in protective immunity against malaria.

The genomic DNAs of bacteria and vertebrates differ in the

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frequency and methylation of CpG dinucleotides, which are at the expected random frequency in bacterial DNA (approximately 1 every 16 bases) but are under-represented (CpG suppression) (1 every 50 to 1 every 60 bases) and methylated in vertebrate DNA (3). Bacterial DNA or synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG dinucleotides in particular base contexts induce B-cell proliferation and immunoglobulin (Ig) secretion, monocyte secretion of the Th1-like cytokines interleukin-12 (IL-12) and alpha, beta, and alpha/beta interferons, gamma interferon (IFN-γ) secretion, and natural killer (NK) cell lytic activity (2, 6, 17, 18, 26, 33, 34). Such immune stimulatory CpGs are typically preceded on the 5′ side by an ApA, GpA, or GpT and followed on the 3′ side by two pyrimidines, especially TpT (CpG motifs) (37). Methylated bacterial DNA or ODNs in which the cytosines of CpG have been converted to 5-methyl-cytosine (the form present in vertebrate DNA) fail to induce immune activation (18). Thus, this simple structural difference between vertebrate and prokaryotic genomic DNAs may function as a "danger signal" to trigger innate immune defenses against infection and initiate a specific immune response (5, 13, 20, 21, 22). Immunization of animals against an antigen using a CpG ODN as an adjuvant induces strong Th1-like responses, as evidenced by potentiated cytotoxic T-cell responses and a preponderance of the IgG2a antibody isotype (7, 23, 30, 32, 34). These responses are antigen specific. In addition to such antigen-specific responses (22), it appears that the strong Th1-like effect of CpG-S motifs can induce nonspecific innate immune responses of a protective nature (13, 21). For example, pretreatment of mice with a CpG-S ODN was demonstrated to provide complete protection against infection with a lethal challenge of the bacteria *Listeria monocytogenes* (19) and *Leishmania major* (39).

Plasmodium yoelii- and *P. falciparum*-infected hepatocytes can be eliminated in vitro by the addition of recombinant IFN-γ to the cultures (11, 24, 25). Parenteral injection of recombinant IL-12 (rIL-12) 2 days before malaria sporozoite challenge completely prevents development of blood-stage infection in mice (31) and monkeys (15). In the BALB/c mouse model, irradiated sporozoite- and DNA vaccine-induced protection is dependent on $CD8^+$ T cells, IFN-γ, IL-12, NK cells, and inducible nitric oxide synthase (iNOS) (8, 9). Since both IL-12 and IFN-γ are induced by immunostimulatory CpG motifs (6, 17), we evaluated whether a CpG ODN might prove beneficial for protection against malaria. We show here that mice challenged with *P. yoelii* sporozoites can be completely protected against malaria sporozoite challenge when pretreated with CpG ODNs and that this protective effect is dependent on IL-12 and IFN-γ.

MATERIALS AND METHODS

ODNs. The CpG ODNs used in this study were 1826 (TCC ATG ACG TTC CTG ACG TT, with the CpG dinucleotides underlined for clarity), a 20-mer which has a nuclease-resistant phosphorothioate backbone and which contains two copies of a CpG motif known to have potent immunostimulatory effects on the murine immune system, and 1585 (gg GGT CAA CGT TGA ggg ggG, with g representing diester linkages and phosphorothioate linkages being to the right of lowercase letters), a 20-mer which contains an unmethylated CpG motif in the middle of a 10-bp palindrome known to induce significant NK cell activity (2, 6). ODNs 1982 (TCC AGG ACT TCT CTC AGG TT) and 2118 (ggG GTC AAG CTT GAg ggg gG) were used as non-CpG control ODNs for the ODN 1826 and 1585 studies, respectively. ODNs were provided by Coley Pharmaceutical Group, Inc. (Wellesley, Mass.). The Na^+ salts of the ODNs were ethanol precipitated, resuspended in phosphate-buffered saline (PBS) (pH 7.2) (ODN 1826) or Tris-EDTA (pH 8.0) (ODN 1585) at a concentration of 4 to 6 mg/ml, and stored at 4°C prior to injection. The endotoxin level in the ODNs was undetectable (less than 1 ng/mg of ODN) by the Limulus assay (Whittaker Bioproducts, Walkersville, Md.).

CpG ODN treatment. All studies were carried out with 4- to 8-week-old female BALB/c ByJ mice (Jackson Laboratory, Bar Harbor, Maine), with 6 to 18 mice in each experimental group. Mice received a single injection into the tibialis anterior muscle of 3, 10, 50, or 100 μg of CpG ODN 1826 or 50, 100, 200, or 500 μg of CpG ODN 1585 in 50 μl of saline at 7, 2, or 1 day(s) prior to sporozoite infection, on the day of infection, and/or at 1 day postinfection. In all experiments, non-CpG control ODNs (1982 or 2118, respectively) were administered in parallel.

Parasites and parasite challenge. *P. yoelii* (17XNL nonlethal strain, clone 1.1) was maintained by alternating passage of the parasites in *Anopheles stephensi* mosquitoes and CD-1 mice. Sporozoites were isolated from mosquito salivary glands 14 days after the mosquitoes had taken an infectious blood meal, by hand dissection of the mosquito salivary glands in M199 medium containing 5% normal mouse serum. The recovered sporozoites were diluted to a final concentration of 250 infectious sporozoites per ml. For infection (day 0), each mouse was injected in the tail vein with 50 *P. yoelii* sporozoites in a volume of 200 μl. It has been established previously that infection with as few as one or two sporozoites of *P. yoelii* 17XNL will result in patent infection of 50% of BALB/c mice. Giemsa-stained blood films were prepared on days 4 to 14 postchallenge and examined microscopically for the presence of parasites, with up to 50 oil immersion fields being examined. Protection was defined as the complete absence of blood-stage parasitemia at all time points.

In vivo depletions. In vivo depletions were carried out as described previously (8, 9). All Igs were purified from ascites (Harlan Bioproducts for Science, Indianapolis, Ind.) by 50% ammonium sulfate precipitation, and final antibody concentrations were determined by optical density readings.

(i) IFN-γ depletion. To deplete IFN-γ, mice received the anti-IFN-γ monoclonal antibody (MAb) XMG-6 (4; kindly provided by F. Finkelman, University of Cincinnati College of Medicine, Cincinnati, Ohio) on each of several days. In the first study, mice received an intraperitoneal (i.p.) injection of 1 mg of the anti-IFN-γ on days −2, −1, and 0 relative to challenge. In the second study, mice received an injection of 1 mg of the anti-IFN-γ MAb on each of days −3 (intravenous [i.v.]), −2 (i.p.), 0 (i.v.), and +2 (i.v.) relative to challenge on day 0.

(ii) IL-12 depletion. To deplete IL-12, mice were injected i.p. with 1 mg of the anti-IL-12 MAb C17.8 (36; kindly provided by M. Wysocka and G. Trinchieri, The Wistar Institute, Philadelphia, Pa.) at 12 h prior to and 3 h after infection.

(iii) Nitric oxide depletion. Aminoguanidine is a competitive substrate inhibitor of iNOS. To deplete nitric oxide, mice were administered 50 mg of aminoguanidine (Sigma Chemical Company, St. Louis, Mo.)/kg of body weight in 0.5 ml of PBS via gastric lavage twice daily, commencing 24 h (experiment 1) or 48 h (experiment 2) before ODN administration and continuing for 72 h (experiment 1) or 96 h (experiment 2) postchallenge.

(iv) NK cell depletion. To deplete NK cells, mice received a single i.v. dose of 200 μl of anti-asialo GM1 antiserum (Wako Bioproducts, Richmond, Va.) diluted 1:8 in 0.5× PBS (25 μl of stock; approximately 675 μg of purified antibody) on days −2, 0, +2, and +4 relative to challenge on day 0.

(v) $CD4^+$ T-cell depletion. The anti-$CD4^+$ MAb GK1.5 (rat IgG2a) was obtained from the American Type Culture Collection (TIB207). On days −7, −6, −5, −4, −3, −2, 0, and +2 relative to challenge on day 0, mice received a single i.p. dose of 1.0 mg of the anti-$CD4^+$ MAb GK1.5 to deplete $CD4^+$ T cells.

(vi) $CD8^+$ T-cell depletion. The anti-$CD8^+$ MAb 2.43 (mouse IgG2a) was also obtained from the American Type Culture Collection (TIB210). On days −5, −4, −3, −2, and 0 relative to challenge on day 0, mice received a single i.p. dose of 0.5 mg of the anti-$CD8^+$ MAb 2.43 to deplete $CD8^+$ T cells.

(vii) Control treatment. In all studies, mice were treated in parallel with a purified rat Ig control (Rockland Co., Gilbertsville, Pa.) in the same manner as the test antibodies.

In vitro assay for IL-12 levels. Blood was obtained from several mice on days 2 to 4 after administration of the ODNs. Sera were separated and frozen at −70°C. Circulating levels of IL-12 (p40) were assayed using a commercially available enzyme-linked immunosorbent assay kit (PharMingen, San Diego, Calif.) according to the manufacturer's specifications.

Statistical analysis. Statistical analysis was performed using the chi-square test (uncorrected) or Fisher's exact test (two-tailed) (if the expected cell value was less than five) (Epi Info, Version 6.04b, Centers for Disease Control and Prevention, Atlanta, Ga.). In all cases, P values of <0.05 were considered significant.

The experiments reported herein were conducted according to the principles set forth in the "Guide for the care and use of laboratory animals," Institute of

TABLE 1. CpG ODN 1828 induces protective immunity against sporozoite challenge

Treatment	Day of ODN administration[a]	No. infected/ total	% Protection	*P* value[b]
CpG ODN 1826	−7	4/10	60	0.005
	−2	0/10	100	0.000008
	−1	0/10	100	0.000008
	0	10/10	0	0.999
Control ODN 1982	−2	10/10	0	
None		18/18	0	

[a] CpG ODN (50 μg) was administered i.m. in the tibialis anterior muscle on the day indicated relative to i.v. inoculation of sporozoites on day 0.

[b] *P* values were calculated by comparing protection induced by CpG ODN 1826 treatment versus control ODN 1982 treatment on each of days −7, −2, −1, and 0 relative to sporozoite challenge on day 0, using the chi-square test (uncorrected) or Fisher's exact test (two-tailed) (if the expected cell value was less than five). *P* values of <0.05 were considered significant.

Laboratory Animal Resources, National Research Council, National Academy Press, Washington, D.C., 1996.

RESULTS

Effect of CpG ODN 1826 on course of *P. yoelii* infection. In all experiments, injection of naïve, untreated mice with *P. yoelii* sporozoites resulted in blood-stage infection (parasitemia) in 100% of mice within 14 days. Pretreatment of mice with CpG ODN 1826 provided complete protection from infection when the CpG ODN was administered 1 or 2 days prior to challenge (Table 1). However, protection was only partial when a longer period (7 days) intervened between the CpG ODN treatment and challenge. In this case, only 30 to 80% of mice were protected (Tables 1 and 2). Mice receiving CpG ODN 1826 at the same time as sporozoite infection were not protected (Table 1). These data suggested that the protective effect of the CpG ODN was mediated by a downstream nonspecific stimulation of the innate immune system but that this effect decreased over time.

Doses of 3 to 100 μg of CpG ODN 1826 gave increasing levels of protection (Table 2), suggesting that the innate immune response stimulated by the CpG ODN was dependent on the amount of CpG ODN administered.

There was usually no protection, and never more than 20% protection, in mice receiving the control (non-CpG) ODN 1982 (Tables 1 and 2). There was no relationship between dose and partial protection with the control ODN, since the group in which some mice did not become infected received 3 μg of control ODN, and all mice which received 10, 50, or 100 μg of control ODN were infected (Table 2).

In total, these findings indicate that the protective effects seen with the CpG ODN 1826 were due to the immunostimulatory effects of the CpG motifs.

Role of IL-12 in protective effect of CpG ODN 1826. Since IL-12 has been implicated with a role in CpG ODN-induced immunity in other systems (17–22), we depleted mice of IL-12 in vivo. There was a complete loss of protection in mice administered CpG ODN 1826 2 days before challenge and additionally treated with anti-IL-12 MAb (Table 3). Administration of rat Ig as a control had no effect on the CpG ODN-induced protection (Table 3). We also assessed circulating IL-12 levels in some of the mice. A single determination of IL-12 levels was done on four mice in the experimental (1826) ODN group and four mice in the control (1982) ODN group (Table 4). Consistent with other reports that CpG ODNs cause IL-12 secretion, enhanced levels of IL-12 were detected in the circulation of mice treated with CpG ODN 1826 (mean, 13,914 pg/ml) compared with mice treated with control ODN 1982 (mean, 1,367 pg/ml) or not treated but infected mice (mean, 389 pg/ml). Thus, the levels of IL-12 in the circulation of CpG ODN 1826-treated mice were at least 10 times greater than background.

Although the pathological consequences of CpG ODN administration were not specifically studied here, there was no evidence of any adverse reactions associated with CpG ODN 1826 (data not presented). However, our recent experience with adverse reactions associated with administration of rIL-12 to humans (unpublished data) but not with IL-12 administration to mice (31) or monkeys (15) indicates that the apparent pathological response of mice and monkeys may not predict outcome in humans.

Role of IFN-γ in protective effect of CpG ODN 1826. CpG ODNs are also known to induce IFN-γ secretion (18–22), suggesting that IFN-γ may be involved in the CpG-induced protection against sporozoite challenge. Therefore, we treated mice with either control antibody or the anti-IFN-γ MAb XMG-6 to specifically deplete IFN-γ in vivo, using the treatment regimen previously shown to be effective in eliminating the protection induced by immunization with irradiated sporozoites or plasmid DNA (8, 9). In the first study, 80% of mice treated with anti-IFN-γ were still protected by administration of CpG ODN 1826 (Table 3, experiment 1). One possibility for the lack of effect of the anti-IFN-γ MAb was the IFN-γ responses were so robust that the MAb was not able to neutralize them. We therefore conducted a second experiment in which the duration of anti-IFN-γ MAb administration was increased and most of the injections were done i.v. Protection was re-

TABLE 2. Dose of CpG ODN 1826 required for protective immunity

ODN	Dose of ODN (μg)	Day of ODN administration[a]	No. infected/ total	% Protection	*P* value[b]
1826	50	−2	0/10	100	0.000008
	100	−7	2/10	80	0.0007
	50	−7	5/10	50	0.033
	10	−7	7/10	30	0.211
	3	−7	7/10	30	0.999
1982	100	−7	10/10	0	
	50	−7	10/10	0	
	10	−7	10/10	0	
	3	−7	8/10	20	
None	0		6/6	0	

[a] CpG ODN was administered i.m. in the tibialis anterior muscle on the day indicated relative to i.v. inoculation of sporozoites on day 0.

[b] *P* values were calculated by comparing protection induced by CpG ODN 1826 treatment versus control ODN 1982 treatment, for each concentration of ODN, using the chi-square test (uncorrected) or Fisher's exact test (two-tailed) (if the expected cell value was less than five). *P* values of <0.05 were considered significant.

TABLE 3. Mechanism of CpG ODN 1826-induced protective immunity

Expt	ODN treatment[a]	Treatment	No. infected/ total	% Protection	*P* value[f]
1	CpG ODN 1826		0/10	100	
	CpG ODN 1826	Control Ig	0/10	100	
	CpG ODN 1826	Anti-IFN-γ[b]	2/10	80	0.474
	CpG ODN 1826	Anti-IL-12	10/10	0	0.000008
	Control ODN 1982		10/10	0	
	None		18/18	0	
2	CpG ODN 1826		0/10	100	
	CpG ODN 1826	Control Ig	0/10	100	
	CpG ODN 1826	Anti-IFN-γ[c]	8/10	20	0.0007
	Control ODN 1982		8/10	20	
	None		6/6	0	
3	CpG ODN 1826	Control Ig	0/9	100	
	CpG ODN 1826	Anti-iNOS[d]	1/9	89	0.999
	CpG ODN 1826	Anti-NK cells	2/9	78	0.474
	Control ODN 1982	Control Ig	8/8	0	
	None		9/9	0	
4	CpG ODN 1826	Control Ig	2/10	80	
	CpG ODN 1826	Anti-iNOS[e]	0/10	100	0.474
	CpG ODN 1826	Anti-$CD8^+$ T cells	1/9	89	0.999
	CpG ODN 1826	Anti-$CD4^+$ T cells	0/10	100	0.474
	Control ODN 1982	Control Ig	nt[g]	nt	
	None		5/10	50	

[a] CpG ODN (50 μg) was administered i.m. in the tibialis anterior muscle on day −2 relative to i.v. inoculation of sporozoites on day 0.
[b] One milligram of anti-IFN-γ MAb was administered i.p. on days −2, −1, and 0 relative to challenge.
[c] One milligram of anti-IFN-γ MAb was administered on each of days −3 (i.v.), −2 (i.p.), 0 (i.v.), and +2 (i.v.) relative to challenge.
[d] Aminoguanidine (50 mg/kg of body weight) was administered twice daily by gastric lavage, commencing 24 h before ODN administration and continuing until 72 h postchallenge.
[e] Aminoguanidine (50 mg/kg of body weight) was administered twice daily by gastric lavage, commencing 48 h before ODN administration and continuing until 96 h post challenge.
[f] *P* values were calculated by comparing protection induced by MAb treatment versus control Ig treatment for each treatment group using the chi-square test (uncorrected) or Fisher's exact test (two-tailed) (if the expected cell value was less than five). *P* values of <0.05 were considered significant.
[g] nt, not tested.

duced from 100 to 20% ($P = 0.0003$; chi-square test), the same level conferred by treatment with the control ODN (Table 3, experiment 2). Administration of rat Ig as an antibody control had no effect on the CpG ODN-induced protection. These data establish that the CpG ODN 1826-induced protection was dependent on IFN-γ as well as IL-12.

Role of NK cells and nitric oxide in protective effect of CpG ODN 1826. Previously, it was reported that the protective immunity against *P. yoelii* sporozoite challenge in BALB/c mice induced by immunization with irradiated sporozoites or plasmid DNA is dependent on nitric oxide and is mediated by $CD8^+$ T cells but not $CD4^+$ T cells, and in part by NK cells (8, 9). Therefore, we next investigated if a similar protective mechanism was induced by treatment with CpG ODN 1826. In two separate experiments, treatment of mice with aminoguanidine, a competitive substrate inhibitor of iNOS, had little or no effect on the ODN 1826-induced protection (Table 3). Likewise, treatment with anti-asialo GM1 antibodies to eliminate NK cells or with MAbs to specifically deplete $CD8^+$ or $CD4^+$ T cells had no significant effect on protection (Table 3).

Although we cannot exclude the possibility that the treatment regimens used here may have been inadequate to deplete a potentially robust immune response induced by the CpG ODN, these data suggested that the ODN-induced protection may be mediated via an effector mechanism distinct from that activated by the irradiated sporozoite or DNA vaccines (9). It is possible that the effector mechanism induced by irradiated sporozoites or DNA vaccines might still have been operative but that an alternate protective mechanism(s) may be activated with CpG ODNs.

Effect of CpG ODN 1585 on course of *P. yoelii* infection. Previous studies in other systems showed that another CpG ODN, 1585, caused preferential activation of NK cells (2, 6). Since NK cells have been implicated with a role in protection against *P. yoelii* sporozoite challenge (9), we next determined whether administration of CpG ODN 1585 could protect against malaria. As shown in Table 5, pretreatment of mice with doses of 50 to 500 μg of CpG ODN 1585 protected 20 to 90% of mice from infection when the CpG ODN was administered around the time of sporozoite challenge. Mice receiving CpG ODN 1585 at the same time as sporozoite infection could be protected, although treatment prior to challenge appeared to be more effective (Table 5). The highest level of protection (90%) resulted from administration of 200 μg of CpG ODN 1585 the day before challenge or 100 μg of CpG ODN 1585 on the day before and the day of challenge (Table 5). Despite different doses and administration regimens, however, we were unable to achieve complete sterile immunity with CpG ODN 1585.

Role of IL-12, IFN-γ, and NK cells in protective effect of CpG ODN 1585. Next, we investigated the mechanism of protection induced by treatment with CpG ODN 1585. As noted with CpG ODN 1826, where protection was dependent on both IL-12 and IFN-γ, in vivo depletion of either IL-12 or IFN-γ significantly reduced the protection conferred by treatment with CpG ODN 1585 (Table 6). Consistent with the reported ability of CpG ODN 1585 to activate NK cells (2) and of NK cells to protect against sporozoite challenge (9), treatment of

TABLE 4. Circulating levels of IL-12 induced by ODN administration

Treatment	Day post challenge[a]	Circulating IL-12 (pg/ml)	Mean (pg/ml) of circulating IL-12
CpG ODN 1826	2	10,087	13,914
	1	12,214	
	0	10,523	
	1	22,831	
Control ODN 1982	2	1,484	1,367
	2	1,538	
	0	1,181	
	1	1,264	
Infectivity control	1	105	389
	1	418	
	1	168	
	1	863	

[a] CpG ODN (50 μg) was administered i.m. in the tibialis anterior muscle on day −2 relative to i.v. inoculation of sporozoites on day 0. Serum was collected from individual mice on the day indicated, relative to i.v. inoculation of sporozoites on day 0, and assayed for IL-12 level by enzyme-linked immunosorbent assay.

TABLE 5. CpG ODN 1585 induces protective immunity against sporozoite challenge

Expt	Treatment	Dose of ODN (μg)	Day of ODN treatment[a]	No. infected/ total	% Protection	*P* value[b]
1	CpG ODN 1826	100	−1	1/10	90	0.0001
	CpG ODN 1585	200	−1	3/10	70	0.003
	CpG ODN 1585	100	−1	6/10	40	0.087
	CpG ODN 1585	50	−1	8/10	20	0.474
	Control ODN 2118	200	−1	10/10	0	
	None			6/6	0	
2	CpG ODN 1826	100	−1	0/10	100	0.000008
	CpG ODN 1585	500	−1	3/10	70	0.003
	CpG ODN 1585	500	0	7/10	30	0.210
	CpG ODN 1585	500	+1	6/10	40	0.087
	CpG ODN 1585	200	−1	1/10	90	0.0001
	CpG ODN 1585	200	0	8/10	20	0.474
	CpG ODN 1585	200	+1	8/10	20	0.474
	CpG ODN 1585	100	−1, 0	1/10	90	0.0001
	CpG ODN 1585	100	−1, 0, +1	1/10	90	0.0001
	Control ODN 2118	500	−1, 0, +1	10/10	0	
	None			10/10	0	

[a] CpG ODN was administered i.m. in the tibialis anterior muscle on the day indicated relative to i.v. inoculation of sporozoites on day 0.

[b] *P* values were calculated by comparing protection induced by CpG ODN 1585 treatment versus control ODN 2118 treatment, using the chi-square test (uncorrected) or Fisher's exact test (two-tailed) (if the expected cell value was less than five). *P* values of <0.05 were considered significant.

mice with anti-asialo GM1 antibodies also had a significant effect on protection (Table 6). Administration of rat Ig control had no effect on the CpG ODN 1585-induced protection.

Role of $CD8^+$ and $CD4^+$ T cells in protective effect of CpG ODN 1585. We also investigated the requirement for nitric oxide and $CD8^+$ and $CD4^+$ T cells in the CpG ODN 1585-induced protection. As shown in Table 6, treatment of mice with aminoguanidine to specifically deplete nitric oxide markedly reduced the protection. Unexpectedly, in vivo depletion of $CD8^+$ T cells with an anti-$CD8^+$ MAb also appeared to reduce the ODN-induced protection (Table 6). Treatment with an anti-$CD4^+$ MAb had no effect. These data suggest that CpG ODN 1585 treatment alone may increase $CD8^+$ T-cell function, including the $CD8^+$ T cell-mediated production of IFN-γ. We consider it unlikely, however, that $CD8^+$ T cells are the primary source of the IFN-γ. In the irradiated sporozoite and *P. yoelii* circumsporozoite protein (PyCSP) DNA vaccine models, we believe that parasite-specific $CD8^+$ T cells are critical for the initial activation of the effector response and that these cells trigger a mechanism of adaptive immunity which is dependent on T cell- and non-T cell-derived cytokines, in particular IFN-γ and IL-12, and requires NK cells but not $CD4^+$ T cells (9).

In total, our data indicate that the protective mechanism induced by administration of CpG ODN 1585 in the absence of parasite antigen is similar in nature to the mechanism induced by immunization with radiation-attenuated *P. yoelii* sporozoites or with plasmid DNA encoding preerythrocytio-stage *P. yoelii* antigens.

DISCUSSION

Our studies to assess the protective role of CpG ODNs against challenge with *P. yoelii* sporozoites were initiated because of an observation made in an experiment in which we used CpG ODN 1826 as an immune enhancer for a DNA vaccine. In that experiment, 100 μg of CpG ODN 1826 was combined with 100 μg of a PyCSP DNA vaccine or the control DNA vaccine without insert, with the intended purpose of using ODNs to enhance the immunogenicity of the DNA vaccine. The ODN-DNA vaccines were administered intramuscularly (i.m.) to 4- to 8-week-old BALB/c ByJ mice in three doses at 3-week intervals. Two weeks after the last dose, mice were challenged with 50 *P. yoelii* sporozoites. In this study, there was no apparent positive effect of the CpG ODN coadministration on the induction of antigen-specific antibodies or cytotoxic T lymphocytes or on protection. However, it was observed that 23% (3 of 13) of the control animals that received the placebo DNA vaccine with the CpG ODN were protected from malaria sporozoite challenge, yet none (0 of 14) of the animals that received just the placebo DNA vaccine were protected (data not shown). This suggested to us that the CpG ODN was inducing a nonspecific protective effect.

Results of subsequent studies presented here clearly show that the stimulation of the innate immune system by CpG immunostimulatory motifs incorporated in either ODN 1826 or 1585 can confer complete protection against malaria in mice. There is a latency period for the development of such immunity, and CpG ODNs must be administered at least 1 day prior to infection for complete protection. Furthermore, the protective effects are relatively short-lived and are already diminishing by 7 days, as evidenced by the decreased protection seen when CpG ODN 1826 is administered 7 days prior to infection. Nevertheless, the finding that with treatment 7 days prior to challenge higher doses of CpG ODNs gave better protection than lower doses indicates that even higher doses may provide longer-lasting protection. A more prolonged antimalaria effect might also be obtained following repeat dosing with a CpG ODN or delivery of a CpG ODN in controlled release vesicles (e.g., microencapsulated) or formulated in such a way as to retard in vivo degradation (e.g., liposomes).

TABLE 6. Mechanism of CpG ODN 1585-induced protective immunity

ODN	Treatment	Days of CpG ODN administration[a]	No. infected/ total	% Protection	*P* value[d]
1826	Control Ig	−1, 0	0/10	100	
1585	Control Ig	−1, 0	3/9	67	
1585	Anti-IFN-γ[b]	−1, 0	8/9	11	0.016
1585	Anti-IL-12	−1, 0	8/9	11	0.016
1585	Anti-iNOS[c]	−1, 0	6/9	33	0.157
1585	Anti-NK cells	−1, 0	7/9	22	0.058
1585	Anti-$CD8^+$ T cells	−1, 0	6/9	33	0.157
1585	Anti-$CD4^+$ T cells	−1, 0	3/9	67	0.999
2118	Control Ig	−1, 0	10/10	0	
None			18/18	0	

[a] ODN 1826 (50 μg) or ODN 1585 (200 μg) was administered on days −1 and 0 relative to i.v. inoculation of sporozoites on day 0.

[b] One milligram of anti-IFN-γ MAb was administered on each of days −3 (i.v.), −2 (i.p.), 0 (i.v.), and +2 (i.v.) relative to challenge.

[c] Aminoguanidine (50 mg/kg of body weight) was administered twice daily by gastric lavage, commencing 24 h before ODN administration and continuing until 72 h postchallenge.

[d] *P* values were calculated by comparing protection induced by CpG ODN 1585 treatment versus control ODN 2118 treatment, using the chi-square test (uncorrected) or Fisher's exact test (two-tailed) (if the expected cell value was less than five). *P* values of <0.05 were considered significant.

The protective antimalaria effect induced by CpG ODNs appears to be mediated by cytokines, since the protection could be abrogated by treatment with a MAb against IL-12 or IFN-γ. These results are consistent with previous in vivo and in vitro findings. Parenteral injection of rIL-12 into mice (31) or monkeys (15) 2 days before malaria sporozoite challenge completely prevented development of blood-stage infection. Administration of anti-IFN-γ to the mice eliminated the protective effect, and the protection in the monkeys was associated with circulating levels of IFN-γ.

It is presumed that administration of CpG DNA enhances the production of IFN-γ, which in turn induces the intracellular generation of nitric oxide, leading to the destruction of the infected hepatocytes, as has been reported for DNA vaccines and the irradiated sporozoite vaccine (8, 9). This model is supported by in vitro observations that *P. yoelii*- and *P. falciparum*-infected hepatocytes can be eliminated by exposure to IFN-γ (11, 24, 25) and that this activity of IFN-γ is prevented by inhibition of iNOS (25). The data obtained here, at least with CpG ODN 1585, are consistent with this interpretation. The apparent lack of involvement of nitric oxide and NK cells in the CpG ODN 1826-induced protection may simply be a reflection of inadequate depletion of a robust immune response induced by the CpG ODN or of activation of an alternate pathway that can also confer protection. In support of this, a higher level of protection was always induced by the CpG ODN 1826 compared with the CpG ODN 1585. We cannot exclude the possibility that these different CpG ODNs (1826 and 1585) may induce distinct protective mechanisms.

Recently, it has been reported that NKT cells can significantly inhibit the liver-stage development of *P. yoelii* and *Plasmodium berghei* in vivo (12) and in vitro (29). Interestingly, the inhibition of liver-stage development induced by in vivo administration of alpha-galactosylceramide was shown to require NKT cells and CD1 molecules, but not NK cells, T cells, or B cells, and was absolutely dependent on IFN-γ but independent of IL-12 (12). The same researchers reported that the mode of protection mediated by the activated NKT cells was distinct from that induced by administration of rIL-12, since neither NKT cells nor CD1 were required for the antimalarial activity of IL-12. Our data demonstrating an absolute requirement for IL-12, as well as IFN-γ, and a role for NK cells suggest that the CpG ODN-induced protection is mediated by an IL-12-dependent mechanism which may not involve activated NKT cells. Further experiments are required to confirm this.

From a practical point of view, a brief period of protection from malaria may be adequate for persons passing through or spending short periods of time in areas where malaria is endemic. Even in cases where protection for longer than a week is desired, it may be possible to give repeat administrations of CpG ODNs. In this event, CpG ODNs may prove simpler, less expensive, and safer to use than repeated administrations of cytokines, although the potential side effects associated with administration of any nonspecific proinflammatory stimulus would need to be considered.

It is possible that longer-lasting and more robust protection could be attained by coupling the CpG ODN-induced protective innate responses with antigen-specific responses, with a potentially lower dose of the CpG ODN being required compared to that required for direct prophylaxis. It has been shown with a number of antigens, including the *Py*CSP (16), that CpG ODNs are potent adjuvants for the induction of Th1-type immunity as well as antibody responses (7, 16, 23, 26, 30, 32, 34, 39). Similar effects may also be realized when CpG ODNs are used as adjuvants with DNA vaccines. Further studies will be necessary to evaluate these possibilities.

ACKNOWLEDGMENTS

The authors thank HM2 Arnell Belmonte, HM3 Romeo Wallace, and Stephen Matheny for providing *P. yoelii* sporozoites and technical assistance. We also thank Tonette Bangura and Norma Graber for technical support, Maria Wysocka and Giorgio Trinchieri (Wistar Institute, Philadelphia, Pa.) for providing the anti-IL-12 MAb C17.8, and Fred Finkelman (University of Cincinnati College of Medicine, Cincinnati, Ohio) for providing the anti-IFN-γ MAb XMG-6.

These studies were supported by the Naval Medical Research and Development Command work units STO F 6.1 61102AA0100BFX and STO F 6.2 62787A00101EFX. A.M.K. is supported by DARPA, the Department of Veterans Affairs, and the National Institutes of Health. H.D. is supported in part by an Ontario Ministry of Health Career Scientist Award.

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Editor: J. M. Mansfield