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Immunostimulatory oligodeoxynucleotides promote protective immunity and provide systemic therapy for leishmaniasis via IL-12- and IFN-γ-dependent mechanisms

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ABSTRACT **Resistance to murine leishmaniasis correlates with development of a CD4$^+$ T helper 1 (Th1)-predominant immune response. To determine whether immunostimulatory CpG-containing oligodeoxynucleotides (CpG-ODN), known to promote a Th1 immune response, could provide protection from *Leishmania* infection, CpG-ODN and freeze-thawed (F/T) *Leishmania major* were coinjected intradermally into susceptible BALB/c mice. A *Leishmania*-specific Th1-predominant immune response was induced, and 40% of animals were protected from subsequent challenge with infectious organisms, with 0% protection of animals injected with F/T *Leishmania* organisms and PBS, F/T organisms and control ODN, or F/T organisms alone. More striking protection (65–95%) was seen in mice first infected with intact *Leishmania* organisms and then injected with CpG-ODN, either at the site of infection or at a remote site. To determine whether the therapeutic protection provided by CpG-ODN depended on IL-12 and IFN-γ production, both IFN-γ-deficient BALB/c mice and BALB/c mice treated with neutralizing anti-IL-12 mAb were first inoculated with *Leishmania* and then treated with either CpG-ODN, ODN, or PBS. None of these IFN-γ-deficient mice survived (0/20, 0/20, and 0/20 respectively). Furthermore, neutralization of IL-12 completely abolished the therapeutic protection provided by CpG-ODN (0/20 mice surviving). We conclude that immunostimulatory DNA sequences likely exert systemic effects via IL-12 and IFN-γ-dependent mechanisms and hold considerable promise as both vaccine adjuvants and potential therapeutic agents in the prevention and treatment of leishmaniasis.**

Infection with the protozoan *Leishmania* is a major health problem with significant morbidity and mortality worldwide. Cellular immune mechanisms are critical for recovery from leishmaniasis and for protection from reinfection in both humans and mice. The immunology of *Leishmania major* infection, a causative agent of cutaneous leishmaniasis, has been well characterized in inbred strains of mice (1–3). Development of a T helper 1 (Th1) (IL-2 and IFN-γ)-predominant CD4 T cell response correlates with resistance in C3H and C57BL/6 mice, whereas the immune response in susceptible BALB/c mice is characterized by production of the Th2 cytokines IL-4 and IL-5 (4–6). Depletion of IFN-γ either by mAb neutralization or gene disruption renders resistant mice susceptible (1). Furthermore, vaccination of susceptible mice with *Leishmania* antigens and recombinant IL-12 or inhibition of endogenous IL-4 confers resistance to *Leishmania* infection in genetically susceptible animals (5, 7). These studies suggest that protection from leishmaniasis results from a Th1 immune response to *Leishmania* antigens.

Although there is currently no immunoprophylactic regimen for leishmaniasis, genetic immunization with plasmid DNA holds promise. In genetic immunization, plasmid DNA encoding pathogen proteins is introduced directly into naive individuals (intradermally or intramuscularly) and a Th1-type immune response is preferentially elicited against the encoded antigen (8, 9). Genetic vaccination with plasmid DNA encoding conserved *Leishmania* proteins, such as the cell-surface glycoprotein gp63 and the LACK protein, has been demonstrated to induce a Th1-type immune response and protective immunity in susceptible BALB/c mice (10–12). In intradermal genetic immunization, cutaneous dendritic cells may take up plasmid DNA and express encoded proteins endogenously (13) or they may acquire gp63 proteins or peptides synthesized by other epidermal or dermal cells. In either case, the ability of relevant accessory cells (e.g., dendritic cells) to stimulate development of protective immunity may be dependent on the adjuvant properties of immunostimulatory sequences that are present on plasmid DNA (14, 15).

Plasmid DNA immunostimulatory sequences contain nonmethylated CpG dinucleotides in a purine-purine-C-G-pyrimidine-pyrimidine motif and preferentially elicit a Th1-type immune response by stimulating production of IL-12 and IFN-α, -β, and -γ (16–18). Oligodeoxynucleotides containing similar immunostimulatory, nonmethylated CpG dinucleotide sequences (CpG-ODN) also induce B cell proliferation and Ig production, monocyte cytokine secretion, and activation of natural killer (NK) cytotoxic activity and IFN-γ release (16, 18, 19). *In vivo*, coadministration of protein vaccines and CpG-ODN facilitates a Th1-type cellular immune response, whereas immunization with conventional protein vaccines alone typically results in a Th2-type immune response with high titers of neutralizing antibodies and poor cellular immunity (18). In aggregate, these data suggest that in the setting of genetic vaccination, immunostimulatory sequences in plasmid DNA (or in CpG-ODN) function as an adjuvant and promote a Th1 immune response to the antigen encoded by the plasmid. We hypothesized that coadministration of CpG-ODN (that contain immunostimulatory sequences) with individual *Leishmania* antigens (or mixtures of antigens) that normally are not protective might promote a Th1 immune response and confer protection.

In the studies described here, several CpG-ODN were characterized with respect to their ability to protect susceptible

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Abbreviations: ODN, oligodeoxynucleotide; F/T, freeze-thawed; NK, natural killer.

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BALB/c mice from *Leishmania* infection. We observed that coinjecting CpG-ODN with unfractionated killed parasites induces IFN-γ production and protective immunity in susceptible BALB/c mice. These studies suggest a potential therapeutic role for CpG-ODN in the prevention or treatment of leishmaniasis and demonstrate that the beneficial effects of CpG-ODN are IL-12- and IFN-γ-dependent in this model.

MATERIALS AND METHODS

Animals. Female BALB/c and C57BL/6 mice were obtained from the National Institutes of Health animal production facility (Frederick, MD). Mice were used at ages ranging from 8 to 12 weeks. All animals were housed and used in experiments in accordance with institutional guidelines. Female BALB/c mice with a targeted mutation of the IFN-γ gene (BALB/c-Ifng-tm1Ts) were obtained from The Jackson Laboratory (stock no. 002286).

Parasites and Parasite Infections. *Leishmania major* (WR 1075) amastigotes were harvested from the hind foot of previously infected BALB/c/J mice. After harvesting, 1×10^5 amastigotes were seeded in flasks containing RPMI 1640 medium supplemented with 20% heat-inactivated FBS. At stationary phase, 6–9 days postinoculation, the promastigotes were removed from the medium by centrifugation at $900 \times g$ for 10 min. After the supernatant was decanted, the pellet was resuspended in 15 ml PBS and the centrifugation-decanting procedure was repeated. The pellet then was resuspended in PBS at 1×10^7 promastigotes/ml. Mice were infected by a s.c. injection of 1×10^6 promastigotes (in 0.1 ml) into the dorsal side of the right hind foot.

Oligodeoxynucleotides. Oligonucleotide DNA sequences used are listed in Table 1. All oligodeoxynucleotides were synthesized with a nuclease-resistant phosphorothioate backbone by Oligos Etc. (Wilsonville, OR), a GMP facility. The ODN were ethanol-precipitated as Na^+ salts and then resuspended in 10 mM Tris, pH 7.0/1 mM EDTA for storage at −20°C and had undetectable lipopolysaccharide levels by *Limulus* assay (BioWhittaker).

Spleen Cell Proliferation Assay. BALB/c spleen B cells (1×10^5 cells in 100 μl/well) were treated with medium or CpG-containing ODN, as indicated, for 48 h at 37°C as described previously (20). Cells were pulsed for the last 4 h with either [^{3}H]thymidine or [^{3}H]uridine (1 μCi/well). Amounts of incorporated ^{3}H were measured by liquid scintillation.

Cytokine ELISA Assays. The concentrations of IL-6, IL-12 p40, tumor necrosis factor α, and IFN-γ in culture supernatants were determined as described previously (17, 20–23). Briefly, supernatants from either BALB/c spleen cells incubated with the different ODN or lymphocyte suspensions cultured with freeze-thawed (F/T) *Leishmania* antigen (see below) were diluted serially, and cytokine concentrations were quantitated at the indicated time points. For ELISA assays, Immulon 22 plates (Dynatech) were coated overnight with the primary anticytokine antibody (1–2 μg/ml), blocked with PBS/10% BSA and washed, incubated with supernatants from ODN-treated splenocytes and washed, treated with biotinylated anticytokine antibodies and washed, and then treated with avidin-conjugated alkaline phosphatase antibodies or avidin-conjugated horseradish peroxidase antibodies, followed by a colorimetric assay with the appropriate substrate. Cytokine concentrations were determined from standard curves generated from recombinant cytokines as described previously (17, 21).

Lymph Node Cell Suspension and Culture Medium. Briefly, draining lymph nodes were removed from mice injected intradermally with F/T killed parasites (16×10^4 parasites per injection) alone or in combination with oligonucleotide DNA 9 days earlier. Lymphocytes were suspended in RPMI 1640 medium supplemented with 10% FCS (Biofluids, Rockville, MD)/2 mM glutamine/1% penicillin/streptomycin/fungizone/10 mM Hepes/1% nonessential amino acids/1 mM sodium pyruvate (all from Life Technologies, Gaithersburg, MD)/5×10^{-5} M 2-mercaptoethanol (Sigma) and incubated in 96-well flat-bottomed microtiter plates (Costar) at 5×10^5 cells per well and cultured with *Leishmania* lysate (0–10 parasite equivalents per cell) for 3 (cytokine assays) or 4 (proliferation assays) days, as described below.

Lymph Node Proliferation Assays. Lymph node cells were cultured for 4 days as described, [^{3}H]thymidine (1 μCi/well) was added during the last 16 h of culture, and cell-associated radioactivity was determined by direct beta counting.

Immunization of Mice and Assessment of Footpad Swelling. Mice were injected intradermally in the ear with F/T killed parasites (16×10^4 parasites/infection) in combination with ODN 1826 or 1911 or PBS (final volume of 100 μl). All animals were boosted at 3 weeks and challenged at 6 weeks with promastigotes as described above. The footpad-swelling assay is the standard measurement of susceptibility to *Leishmania* disease in mice. The progress of infection was assessed by measuring the thickness of the infected footpad weekly by using a dial micrometer (L.S. Starett Co., Athol, MA) as described previously (5, 24) The lesion size was calculated by subtracting the thickness of the uninfected contralateral footpad from that of the infected one (data are expressed as difference in footpad size). Animals were euthanized either when they became unable to eat, drink, or ambulate secondary to their lesions or when the lesions became ulcerated and/or necrotic. All measurements and decisions to euthanize were made by an animal technician blinded to treatment groups.

Therapeutic Application of Oligodeoxynucleotide DNA. Mice were infected with *L. major* promastigotes as described above, and 4 h after infection mice were given a second intradermal injection of either ODN or PBS control in either the site of infection, the contralateral footpad, or ear. The progression of infection was assessed as described above.

Treatment of Mice with Neutralizing Antibodies. Purified neutralizing mAb (rat IgG2a) against murine IL-12 (hybridoma C17.8) was obtained as ascites from G. Trinchieri (Wistar Institute) and Harlan Bioproducts for Science (Madison, WI). Ammonium sulfate-precipitated Ab was injected i.p. (1 mg per mouse) 1 day before *Leishmania* infection and then weekly for 5 weeks. Control mice were treated with normal rat IgG (Sigma).

RESULTS

Comparison of B Cell Proliferation and Cytokine Production by ODN. Before initiating studies to assess the immunomodulatory properties of ODN in murine leishmaniasis, we characterized their effects on lymphoid cells *in vitro*. Each of the different ODN evaluated, except for the control ODN 1911 (Table 1), contain two immunostimulatory CpG motifs (5′-purine-purine-cytosine-guanine-pyrimidine-pyrimidine) in either overlapping or tandem repeats (25, 26). When CpG-ODN 1758, 1826, and 1835 and ODN 1911 were incubated with BALB/c splenocytes, CpG-ODN 1826 (0.6 μg/ml) induced ≈10-fold-greater B cell proliferation and ≈3-fold-more IL-12 and IFN-γ release than CpG-ODN 1758 (0.6 μg/ml) (Table 1). CpG-ODN 1835 and ODN 1911 were significantly less active in all respects (Table 1). Note that CpG-ODN 1826 also induced significantly more IL-6 and tumor necrosis factor α production by BALB/c splenocytes than the other ODN.

IFN-γ Production Is Stimulated by Coinjection of CpG-ODN and *Leishmania* Antigens. To determine whether a *Leishmania*-specific Th1 cytokine response could be induced by coadministering ODN and *Leishmania* antigens, susceptible BALB/c mice were coinjected intradermally with CpG-ODN 1826, 1758, or 1835 or ODN 1911 and F/T *Leishmania* lysate (16×10^4 parasites per ear). Nine days later, draining lymph

Table 1. ODN sequences and differential immune effects of different CpG motifs

ODN (μg/ml)	DNA sequence (5′ to 3′)	B cell proliferation, (1×10^5)/48 h	mIL-6, pg/ml; 24 h/ (2×10^6 cells)	mIL-12, pg/ml; 24 h/ (2×10^6 cells)	IFN-γ, pg/ml; 48 h/ (2×10^6 cells)	TNF-α, pg/ml; 4 h/ (2×10^6 cells)
None		Baseline	ND	879 ± 978	189 ± 132	ND
1911 (6 μg/ml)	TCCAGGACTTTCCTCAGGTT	7-fold	ND	6,697 ± 1,589	1,158 ± 164	ND
1911 (0.6 μg/ml)		3-fold	ND	925 ± 110	113 ± 22	ND
1835 (6 μg/ml)	TCTCCCAG**CG**AG**CG**CCAT	4-fold	46 ± 65	5,596 ± 958	407 ± 184	ND
1835 (0.6 μg/ml)		Baseline	ND	2,452 ± 267	366 ± 174	ND
1826 (6 μg/ml)	TCCATGA**CG**TTCCTGA**CG**TT	92-fold	7,975 ± 356	24,032 ± 1,432	8,965 ± 1,160	1,803 ± 121
1826 (0.6 μg/ml)		75-fold	5,682 ± 365	29,953 ± 2286	11,378 ± 639	579 ± 130
1758 (6 μg/ml)	TCTCCCAG**CG**TG**CG**CCAT	47-fold	2,141 ± 895	13,331 ± 436	6,299 ± 654	286 ± 224
1758 (0.6 μg/ml)		7-fold	ND	8,207 ± 725	4,233 ± 1,974	ND

Assays were performed by incubating the different ODNs with splenocyte cultures as described in *Materials and Methods*. Data represent the mean ± SD of triplicate assays and are representative of 3–12 different experiments that gave similar results. TNF-α, tumor necrosis factor α. ND, not detected.

nodes were removed and the lymph node cells subsequently were cultured with F/T parasites to determine whether *Leishmania*-specific T cell proliferation could be detected and to assess IFN-γ and IL-4 production. Lymph node cells from mice injected with ODN and F/T *Leishmania* lysate did not exhibit increased *Leishmania*-specific T cell proliferation when compared with cells from mice injected with F/T lysate alone (Fig. 1*A*). However, coadministration of ODN did alter IFN-γ production by draining lymph node cells that subsequently were cultured with F/T *Leishmania* lysate *in vitro* (Fig. 1*B*). In particular, coinjection of CpG-ODN 1826 and F/T *Leishmania* lysate augmented IFN-γ production by 2- to 8-fold as compared with injection of F/T *Leishmania* lysate alone (Fig. 1*B*). Interestingly, the coinjection of F/T parasites and CpG-ODN 1758 or 1835 (each with overlapping CpG dinucleotide motifs) resulted in a consistent reduction of IFN-γ production, whereas coadministration of the control ODN 1911 (which is composition-matched to 1826 but lacks a CpG dinucleotide motif) had no effect (Fig. 1*B*). Coinjection of ODN with *Leishmania* lysate did not alter the levels of IL-4 produced by the draining lymph node cells when stimulated subsequently with F/T parasite *in vitro* (Fig. 1*C*).

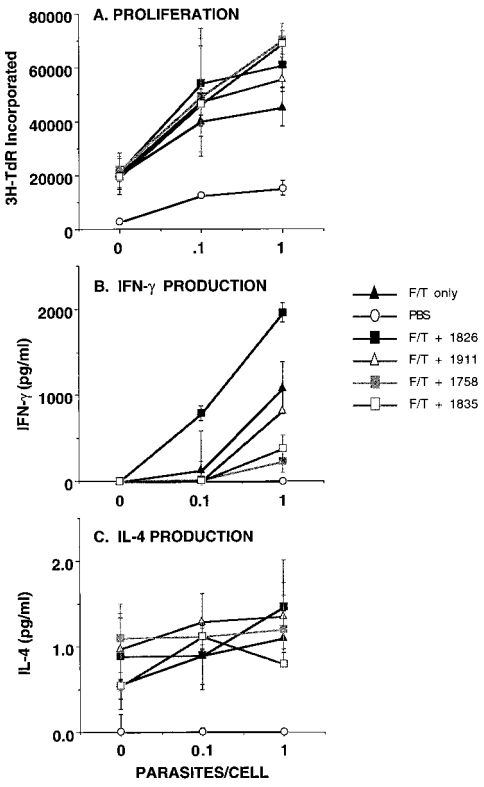

FIG. 1. Coinjection of CpG-DNA and *Leishmania* lysate stimulates IFN-γ production. Lymph node cell suspensions were prepared from BALB/c mice 9 days after their ears were injected with F/T killed parasite lysate alone (▲) or in combination with CpG-ODN 1826 (■), ODN 1911 (△), CpG-ODN 1758 (▨), or CpG-ODN 1835 (□) and assayed for parasite-specific lymphocyte proliferation (*A*), IFN-γ release (*B*), or IL-4 production (*C*), as described in *Materials and Methods*. Results are presented as the mean (± SD) of assays performed in triplicate. The data are representative of three experiments.

Protective Immunity After Vaccination with CpG-ODN and *Leishmania* Lysate. The *in vitro* data suggested that coinjection of CpG-ODN 1826 and *Leishmania* lysate resulted in a Th1-type cytokine/immune response. To determine whether coinjecting CpG-ODN 1826 and F/T *Leishmania* lysate would confer protective immunity, we attempted to vaccinate susceptible BALB/c mice. Mice received intradermal (ear) injection of F/T *Leishmania* lysate alone or in combination with 10 μg of CpG-ODN 1826, control ODN 1911, or PBS. All animals were boosted at 3 weeks by intradermal injection at the same site and challenged at 6 weeks with 10^6 *L. major* parasites injected s.c. (footpad). The progress of *Leishmania* infection was assessed via weekly, blinded measurements of footpad swelling. In two separate experiments (10 animals in each group), protection from *Leishmania* infection was seen in 40% (8/20) of BALB/c mice vaccinated with CpG-ODN 1826 and F/T *Leishmania* lysate, whereas none of the BALB/c mice receiving control ODN 1911 and F/T *Leishmania* lysate (0/20), F/T *Leishmania* lysate alone (0/20), or PBS (0/20; data not shown) were protected (Fig. 2 depicts representative data from one experiment).

No Additional Protection from *Leishmania* When CpG-ODN Are Combined with Plasmid DNA Vaccination. Previously, we found protection from *Leishmania* infection in approximately 30% of BALB/c mice immunized with plasmid DNA encoding the *Leishmania* cell-surface glycoprotein gp63 (12). To determine whether CpG-ODN 1826 could provide an additional protective adjuvant effect, we coinjected CpG-ODN 1826 with plasmid DNA encoding gp63. No additional protection was seen in the BALB/c mice receiving the plasmid DNA (100 μg) and CpG-ODN 1826 (10 μg) combination (3/10 mice) compared with animals that were administered plasmid DNA (100 μg) alone (4/10 mice) (data not shown). This suggests that CpG-ODN do not confer adjuvant effects in addition to those already induced by the immunostimulatory sequences or nonmethylated CpG sequences present in bacterial plasmid DNA.

CpG-ODN Have Therapeutic Potential for the Treatment of Leishmaniasis. Because coinjection of CpG-ODN 1826 and *Leishmania* lysate induced a Th1-type cytokine response and provided partial protection from *Leishmania* infection in susceptible mice, we wanted to determine whether CpG-ODN 1826 would have a therapeutic effect when given after *Leish-*

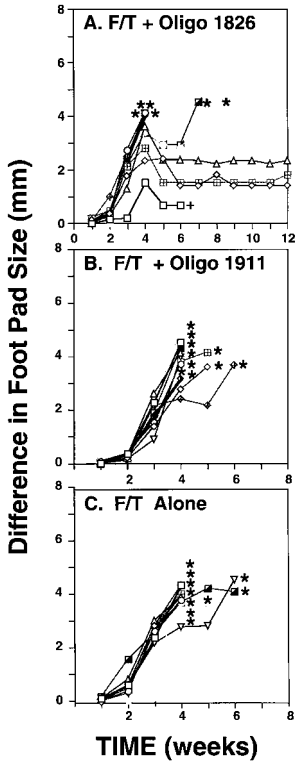

FIG. 2. Vaccination with CpG-DNA and *L. major* lysate is protective. BALB/c mice ears were injected with F/T parasite lysate alone (*C*) or in combination with CpG-ODN 1826 (*A*) or control ODN 1911 (*B*) as described in *Materials and Methods* and subsequently challenged with infectious organisms. Footpad thickness was assessed weekly. Each line represents the progression of infection in an individual animal. *, Euthanized animals. **+**, A single, protected animal that died because of a cage accident. These data are representative of two independent experiments.

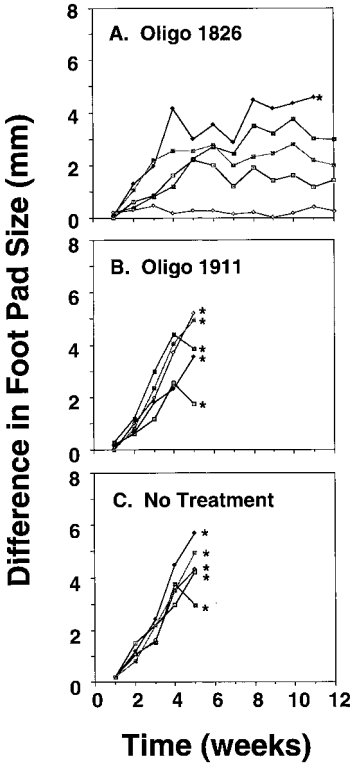

FIG. 3. CpG-DNA has therapeutic potential for the treatment of leishmaniasis. The footpads of BALB/c mice were infected with parasite 4 h before injection with CpG-ODN 1826 (*A*), control ODN 1911 (*B*), or PBS (*C*) as described in *Materials and Methods*. Each line represents the progression of infection in an individual animal. *, Euthanized animals. These data are representative of four independent experiments.

mania challenge/infection. Four hours after inoculation of BALB/c mice with 1×10^6 *L. major* promastigote organisms into the right footpad, the site of infection was injected with either CpG-ODN 1826 (10 μg in 100 μl), control ODN 1911 (10 μg in 100 μl), or PBS, and the footpad size was measured weekly (Fig. 3). Prolonged survival in the footpad-swelling assay was seen overall in 65% (13/20) of infected BALB/c mice receiving CpG-ODN 1826 (four of five of the individual mice in Fig. 3*A*), whereas the infected BALB/c mice receiving control ODN 1911 (0/20) or PBS injections (0/20) developed progressive disease requiring euthanization (Fig. 3*B*). Injection of CpG-ODN 1826 into the inoculation site at weekly intervals did not increase survival (data not shown).

The Therapeutic Effects of CpG-ODN Are Dose-Dependent. To determine the optimal therapeutic dose of CpG-ODN 1826 required to prevent progressive infection, different amounts of CpG-ODN 1826 (3 μg/100 μl, 10 μg/100 μl, or 30 μg/100 μl) were injected into BALB/c footpads 4 h after infection with *L. major* promastigotes (1×10^6 per footpad) (Fig. 4). The therapeutic effect of CpG-ODN 1826 was dose-dependent with a maximal protective effect observed at 10 μg/100 μl (65% survival at 12 weeks). By comparison, CpG-ODN 1826 doses of 30 μg/100 μl and 3 μg/100 μl resulted in the survival of only 10% (2/20) and 20% (4/20) of infected BALB/c mice, respectively.

The Therapeutic Effects of CpG-ODN Are Systemic. Because the CpG-ODN 1826 was injected directly into the infected footpad, it was possible that its effect was limited to the site of infection. To determine whether injection of CpG-ODN 1826 also exerted systemic therapeutic effects, CpG-ODN 1826 (10 μg), ODN-1911 (10 μg), and PBS were injected into the noninfected left footpad after *Leishmania* inoculation into the right footpad (Fig. 5). In parallel, the ODN also were injected into the infected footpad (right footpad) after inoculation of *Leishmania*. Injection of CpG-ODN 1826 into the opposite left (uninfected) footpad resulted in enhanced survival in 70% (14/20) of *Leishmania*-infected animals at 8 weeks. By comparison, injection of CpG-ODN 1826 into the same right (infected) footpad yielded a 70% (14/20) protection rate at 8 weeks whereas no mice injected with control ISS-ODN 1911 into either footpad survived.

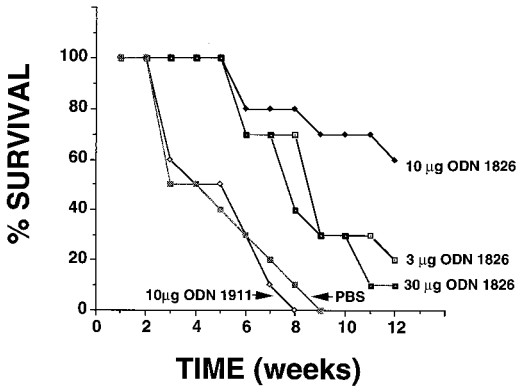

FIG. 4. Therapeutic effects of CpG-DNA are dose-dependent. Footpads of BALB/c mice were infected with *L. major* parasite 4 h before injection with 3 μg (○), 10 μg (◆) and 30 μg (■) CpG-ODN 1826, 10 μg control ODN 1911 (◇), or PBS (▨). Results after injection of 3 μg and 30 μg of ODN 1911 were no different than for 10 μg (data not shown). Each line represents the percentage of animals surviving infection at the indicated time. These data are pooled from two independent experiments; $n = 20$ mice per treatment group.

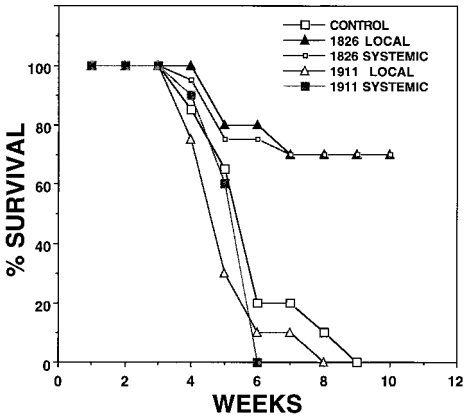

FIG. 5. Therapeutic effects of CpG-ODN are systemic. Footpads of BALB/c mice were infected with *L. major* parasites 4 h before injection with CpG-ODN 1826 in the infected right footpad (◆), CpG-ODN 1826 at a distant site (■), control ODN 1911 in the infected foot (◇), ODN 1911 at a distant site (▨), or PBS (□) as described in *Materials and Methods*. Each line represents the percentage of animals surviving infection at the indicated time. These data are pooled from two independent experiments; $n = 20$ mice per treatment group.

The Mechanism of CpG-ODN Action Is Dependent on IL-12 and IFN-γ Expression. Although we have demonstrated that CpG-ODN induce IL-12 and IFN-γ expression in splenocyte cultures (Table 1), we wanted to determine whether the therapeutic protection provided by ODN 1826 depends on IL-12 and IFN-γ production. IFN-γ-deficient BALB/c mice (BALB/c-Ifng-tm1Ts) were treated with CpG-ODN 1826, ODN 1911, and PBS 4 h after inoculation with *L. major* promastigotes. None of these IFN-γ-deficient mice survived (0/20, 0/20, and 0/20, respectively; Table 2). In the same experiment, 19 of 20 (95%) of the control BALB/c mice infected with *L. major* and treated with CpG-ODN 1826 survived (Table 2). This demonstrates that IFN-γ is essential for the protective effects of ODN 1826, consistent with the critical role of IFN-γ in the Th1-predominant protective immunity against *Leishmania*.

We next assessed the role of IL-12, an important initiator of Th1 immune responses, in the protective mechanism of CpG-ODN 1826. IL-12 was neutralized in BALB/c mice by i.p. injection of anti-IL-12 mAbs 1 day before infection with *L. major* and treatment with CpG-ODN 1826. Neutralization of IL-12 completely abolished the therapeutic protection provided by ODN 1826 (0/20 mice surviving; Table 3). In the same experiment, control BALB/c mice that received i.p. injections of either rat IgG or PBS before infection with *L. major* and treatment with CpG-ODN 1826 had survival rates of 17/20 (85%) and 16/20 (80%), respectively. As expected, the neutralization of IL-12 in C57BL/6 mice (Table 3) that normally are resistant to *Leishmania* infection decreased survival to only 2/15 (13%) of infected mice. These results demonstrate that IL-12 also plays a critical role in the therapeutic protection provided by CpG-ODN 1826.

Table 2. CpG-ODN are ineffective therapy for leishmaniasis in IFN-γ-deficient mice

Treatment group	Survival at 10 weeks
BALB/c-Ifng-tm1Ts (IFN-γ-deficient mice) injected with *L. major* parasites	
1. CpG-ODN 1826	0/20 (0%)
2. CpG-ODN 1911	0/20 (0%)
3. PBS	0/20 (0%)
BALB/c + *L. major* parasites	
1. CpG-ODN 1826	19/20 (95%)
2. CpG-ODN 1911	2/20 (10%)
3. PBS	2/20 (10%)

The footpads of IFN-γ-deficient BALB/c mice and normal BALB/c mice were infected with *L. major* parasites 4 h before injection with CpG-ODN 1826, control ODN 1911, or PBS as described in *Materials and Methods*. Animals were euthanized if footpad thickness decreased mobility or if ulceration and necrosis developed.

Table 3. Therapeutic effects of CpG-ODN depend on IL-12 production

Treatment group	Survival at 6 weeks
BALB/c injected with *L. major* parasites	
1. CpG-ODN 1826 + anti-IL12 Ab	0/20 (0%)
2. CpG-ODN 1826 + rat IgG	17/20 (85%)
3. CpG-ODN 1826 + PBS	16/20 (80%)
4. PBS + PBS	4/20 (20%)
C57BL/6 + *L. major* parasites	
1. Anti-IL-12 Ab	2/15 (13%)
2. Rat IgG	13/15 (87%)
3. PBS	9/10 (90%)

After i.p. injection of anti-IL-12 mAb, rat IgG, or PBS, the footpads of mice were infected with *L. major* parasites and then injected with ODN 1826 as described in *Materials and Methods*. Animals were euthanized if footpad thickness decreased mobility or if ulceration and necrosis developed.

DISCUSSION

Several previous studies demonstrated that genetic immunization with plasmids encoding *Leishmania* antigens induced Th1-predominant immunity and conferred protection (or partial protection) of BALB/c mice from subsequent challenge with *Leishmania* organisms (10–12). In this study, we demonstrate that coinjection of CpG-containing DNA, in the form of CpG-ODN 1826, with *Leishmania* lysate elicited a Th1-type cytokine response and converted *Leishmania* lysate from a nonprotective vaccine into one that was protective in a significant percentage of mice. Injection of CpG-ODN 1826 into BALB/c shortly after inoculation with live *Leishmania* parasites also conferred protection from progressive infection, suggesting that CpG-ODN 1826 can redirect the harmful immune response elicited by live *Leishmania* parasites and that CpG-ODN such as 1826 might be efficacious in the treatment of early leishmaniasis. The level of protection observed with CpG-ODN immunotherapy is comparable to that observed (72–100%) when rIL-12 protein is injected with Leishmania antigens (5, 27–29). CpG-ODN 1826 apparently exerts systemic effects because injection of 1826 into a distant site was as effective as injection into the site of *Leishmania* inoculation. The protective effect of CpG-ODN 1826 depends on IL-12 and IFN-γ production, cytokines that initiate and mediate Th1 immune responses, respectively.

Specific CpG sequences appear to be important for elicitation of Th1-type immunity and enhancement of vaccine efficacy. Even though CpG-ODN 1826 and 1758 both induced significant cytokine responses *in vitro*, only CpG-ODN 1826 promoted a *Leishmania*-specific, Th1-type cytokine response *in vivo*, and CpG-ODN 1758 and 1835 actually reduced IFN-γ production to levels below that seen with control (CpG-lacking) ODN 1911. One notable difference between the effective CpG-ODN 1826 and the ineffective CpG-ODN 1758 and 1835 was that the latter two contained overlapping CpG sequence motifs. Because these CpG-ODN have immunostimulatory activities in other experimental systems, e.g., 1758 is a known activator of NK cells (26), these results suggest that optimal CpG-ODN sequences may be model- or disease-dependent and, at this point, need to be determined empirically in the appropriate *in vivo* model. As our understanding about the mechanisms of action of various CpG-ODN improves, it should be possible to predict effects on immune

responses *in vivo* based on the results of carefully selected *in vitro* assays. At the present time, *in vitro* assays may be most useful in initially screening CpG-ODN for immunostimulatory activity, to determine which CpG-ODN should be assessed further and optimized *in vivo*.

It is not clear why only 40% of animals vaccinated with CpG-ODN and *Leishmania* lysate were protected from *Leishmania* infection. Individual mice immunized with ODNs and *Leishmania* lysate each demonstrated *Leishmania*-specific lymphocyte proliferation and IFN-γ production in response to parasites *in vitro* (data not shown), suggesting that the failure to protect was not secondary to inconsistent priming of recipients. Because the dose-response studies indicated that the therapeutic window was narrow, with higher doses (30 μg) of CpG-ODN 1826 demonstrating decreased protection (see Fig. 4), it is possible that individual animals did not receive optimal protective doses of CpG-ODN 1826. This seems unlikely because animals in each experiment were age- and sex-matched, were essentially the same size, and were inoculated in comparable sites by the same investigator. We also do not understand why CpG-ODN 1826 provided better protection when combined with live *Leishmania* parasites than with *Leishmania* lysate. We hypothesize that this difference may reflect the propensity of live *Leishmania* parasites to infect accessory cells (such as Langerhans cells) that subsequently are involved in T cell priming (30, 31).

The mechanisms of action of CpG-ODN 1826 in the *Leishmania* model that we have studied are being clarified. Although some of the effects that we have observed may be attributable to activation of NK cells, we think that it is more likely that effects of local or systemic administration of CpG-ODN are mediated primarily through accessory cells. We have demonstrated recently that CpG-ODN 1826 activates Langerhans cells (epidermal dendritic cells) *in vitro* and *in vivo* and that a consequence of activation is IL-12 production (15). Consistent with this proposed mechanism, these experiments demonstrate that the effects of CpG-ODN 1826 are dependent on IL-12 and IFN-γ expression. Although increased levels of IL-12 have not been documented in the serum of mice injected intradermally with CpG-ODN 1826, we have demonstrated markedly elevated serum levels of IL-12 in BALB/c mice after i.p. injection of CpG-ODN 1758 (32) and CpG-ODN 1826 (unpublished observations). We hypothesize that, after intradermal CpG-ODN 1826 administration, IL-12 may be produced only by dendritic cells in selected sites such as skin or draining lymph nodes (33).

Our findings suggest that the adjuvant activity of CpG-ODN can be exploited to create protective vaccines composed of complex mixtures of antigens from individual or, perhaps, different pathogens. Consequently, CpG-ODN may be very useful for eliciting humoral and cellular immune responses against a variety of pathogens or for increasing the efficacy of attenuated vaccines that induce partial, but incomplete, protection. Additionally, CpG-ODN adjuvants may be substantially more cost-effective than protein adjuvants, such as rIL-12, because of stability and the cost of production and handling. Vaccine studies involving the use of CpG-ODN in primates are ongoing, and human trials involving the use of ODN in conjunction with defined protein antigens are planned. If the outcome of these early clinical studies is encouraging, it will be reasonable to consider using these interesting reagents as components of vaccines for diseases such as *Leishmania*, leprosy, or tuberculosis. Based on our initial experience in the murine *Leishmania* model, it even may be possible to use CpG-ODN as therapeutic agents in patients with early or limited disease.

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