The Journal of Immunology
RESEARCH ARTICLE | MAY 01 2003

CpG Oligodeoxynucleotides Protect Normal and SIV-Infected Macaques from *Leishmania* Infection [1] FREE

Daniela Verthelyi; ... et. al

J Immunol (2003) 170 (9): 4717–4723.
https://doi.org/10.4049/jimmunol.170.9.4717

CpG Oligodeoxynucleotides Protect Normal and SIV-Infected Macaques from *Leishmania* Infection[1]

Daniela Verthelyi,[2,3]* Mayda Gursel,* Richard T. Kenney,† Jeffrey D. Lifson,‡ Shuying Liu,§ Joan Mican,§ and Dennis M. Klinman*

Oligodeoxynucleotides containing CpG motifs (CpG ODNs) mimic microbial DNA and activate effectors of the innate immune response, which limits the spread of pathogens and promotes an adaptive immune response. CpG ODNs have been shown to protect mice from infection with intracellular pathogens. Unfortunately, CpG motifs that optimally stimulate humans are only weakly active in mice, mandating the use of nonhuman primates to monitor the activity and safety of "human" CpG ODNs in vivo. This study demonstrates that CpG ODN treatment of rhesus macaques significantly reduces the severity of the lesions caused by a challenge with *Leishmania*. *Leishmania* superinfection is common in immunocompromised hosts, particularly those infected with HIV. This study shows that PBMCs from HIV-infected subjects respond to stimulation with CpG ODNs. To determine whether CpG ODNs can protect retrovirus-infected primates, SIV-infected macaques were treated with CpG ODNs and then challenged with *Leishmania*. Both lesion size and parasite load were significantly reduced in the CpG-treated animals. These findings support the clinical development of CpG ODNs as immunoprotective agents in normal and HIV-infected patients. ***The Journal of Immunology,*** **2003, 170: 4717–4723.**

Stimulation of the innate immune system by determinants expressed by infectious microorganisms serves to limit the early spread of a pathogen while promoting the development of Ag-specific immunity (1). Unmethylated CpG motifs present at high frequency in bacterial but not vertebrate DNA are recognized by Toll-like receptor 9 expressed by B cells and plasmacytoid dendritic cells (DCs)[4] (2–4). The interaction of Toll-like receptor 9 with CpG motifs triggers an immune cascade, resulting in improved Ag uptake/presentation by APCs and the secretion of polyreactive Ig, chemokines, and cytokines by B cells, NK cells, DCs, and monocytes (5, 6). Synthetic oligodeoxynucleotides (ODNs) expressing CpG motifs mimic the immunostimulatory activity of bacterial DNA (7).

There is considerable interest in developing novel agents that improve host resistance against infectious microorganisms. Studies in murine models indicate that CpG ODNs facilitate host clearance of infectious pathogens such as *Leishmania*, *Listeria*, and *Francisella tularensis* (8–11). Protection is observed even in T cell-depleted immunodeficient mice, raising the possibility that CpG ODNs might also help immunocompromised patients resist opportunistic infections (9). Exploration of this issue using murine models is of limited value, however, because the precise CpG motifs that are most active in rodents are poorly immunostimulatory in primates (due to evolutionary divergence in CpG recognition) (12–14).

Two types of CpG ODNs that activate PBMCs from human and nonhuman primates have been identified (14–16). "D" type ODNs trigger plasmacytoid DCs to secrete IFN-α (17), monocytes to mature into functionally active DCs (18), and NK cells to secrete IFN-γ (14, 17), whereas "K" type ODNs primarily stimulate B cells and monocytes to proliferate and secrete IgM, IL-10, and IL-6 (15, 17). To date, the ability of these ODNs to stimulate immune cells from immunocompromised donors or to provide protection in vivo in a relevant challenge model has not been examined.

HIV-infected patients have multiple defects in immune reactivity, reflecting a loss in the number and/or function of $CD4^+$ T cells, NK cells, macrophages, and DCs (19–22). These defects increase their susceptibility to opportunistic infections, which in turn accelerates the course of AIDS (23). One such opportunistic pathogen is *Leishmania* (24). Leishmaniasis is a protozoan infection that causes skin lesions ranging in size from small spontaneously healing papules to large mutilating ulcers (24). The course of infection is influenced by the nature of the host's immune response, with Th1-type immunity (high levels of IFN-γ) being associated with reduced parasite load and smaller lesions, whereas Th2-type immunity (increased IL-10) favors more severe disease. HIV-infected patients are more susceptible to infection and typically develop the more aggressive visceral form of the disease (25).

The current study was undertaken to determine whether PBMCs from immunocompromised, retrovirus-infected primates can respond to D and K CpG ODNs. The protective activity of CpG ODNs in *Leishmania*-infected rhesus macaques was then examined (26). Results indicate that CpG ODNs enhance host resistance to infectious challenge by *Leishmania*, even when the subject is immunosuppressed.

*Section of Retroviral Immunology and †Division of Bacterial, Parasitic, and Allergenic Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892; ‡AIDS Vaccine Program, Science Applications International Corp., National Cancer Institute, Frederick, MD 21702; §Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Received for publication October 18, 2002. Accepted for publication February 25, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by Military Interdepartmental Purchase Request MM8926. This project has been funded in part with federal funds from the National Cancer Institute and the National Institutes of Health under Contract NO1-CO-12400. The assertions herein are private ones from the authors and are not to be construed as official or as reflecting the views of the Food and Drug Administration.

[2] Current address: Division of Therapeutic Proteins, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892.

[3] Address correspondence and reprint requests to Dr. Daniela Verthelyi, Building 29A, Room 3B19, 8800 Rockville Pike, Bethesda, MD 20892. E-mail address: Verthelyi@cber.fda.gov

[4] Abbreviations used in this paper: DC, dendritic cell; ODN, oligodeoxynucleotide; i.d., intradermally.

0022-1767/03/$02.00

Materials and Methods

Oligodeoxynucleotides

ODNs were synthesized by the Center for Biologics Evaluation and Research Core Facility. Sequences used were as follows (phosphorothioate bases in uppercase and phosphodiester bases in lowercase): D19, GGtg catcgatgcagGGGGG; D35, GGtgcatcgatgcaggggGG; D29, GGtgcaccggtg cagGGGGG; K3, ATCGACTCTCGAGCGTTCTC; K123, TCGTTTGT TCT; and K23, TCGAGCGTTCTC. Control ODNs included D122 (GGtgcattgatgcagGGGGG) and K163 (TGCAGGCTTCTC). All ODNs had <0.1 endotoxin U of endotoxin per milligram of ODN as assessed by a *Limulus* amebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, MD). Previous studies from our lab have shown that individual humans and monkeys vary in their responses to specific K and D sequences. Indeed, no single D or K motif is optimally stimulatory in all donors (27). However, mixtures of ODNs were identified that strongly stimulated PBMCs from all human donors (15, 27). These D or K ODN mixtures were used in our in vivo studies in macaques.

Human PBMCs

Buffy coats from healthy blood donors were obtained from the National Institutes of Health Department of Transfusion Medicine. PBMCs from HIV-infected subjects were obtained from the Infectious Diseases Section of the Department of Transfusion Medicine at the National Institutes of Health Blood Bank and from the National Institute of Allergy and Infectious Diseases (National Institutes of Health) after appropriate consent. Their clinical characteristics are summarized in Table I.

Rhesus macaques

Healthy 3-year-old rhesus macaques (*Macaca mulata*) were obtained from the Food and Drug Administration colony in South Carolina. All studies were Institutional Animal Care and Use Committee approved and were conducted in an American Association for the Accreditation of Laboratory Animal Care accredited facility. Animals were monitored daily by veterinarians. No systemic or local adverse reactions to CpG ODNs were observed. Treatments were administered and peripheral blood samples obtained from ketamine-anesthetized animals (10 mg/kg, Ketaject; Phoenix Pharmaceuticals, St. Joseph, MD).

Mononuclear cell preparation

Mononuclear cells were isolated by density gradient centrifugation of PBMCs over Ficoll-Hypaque as described (14). Cells were washed three times and cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1.5 mM L-glutamine, and 100 U/ml penicillin/streptomycin at 5×10^5 cells/well in the presence of 1–3 μM ODN. Supernatants were collected after 72 h and tested by ELISA for cytokine and Ab levels.

Macaque treatment groups and protocol

Study no. 1. Eighteen healthy rhesus macaques (six per group) were challenged on the forehead on day 0 with 10^7 *Leishmania amazonensis* (PH8) metacyclic promastigotes intradermally (i.d.) as previously described (15, 28). Three days before and 3 days after challenge, 500 μg of a mixture of K or D ODNs was administered i.d. at the same site. Control monkeys ($n = 6$) received saline. Animals developed a typical self-limited lesion in situ, characterized by erythema, induration, and ulceration. Lesion size, which reflects the severity of infection, was measured weekly in a blinded fashion.

Study no. 2. Fourteen rhesus macaques chronically infected with SIVmac239 were obtained by transfer to the current study after completion of a separate research protocol fully described by Lifson et al. (29). Six healthy macaques were included in the study as controls. The macaques were superinfected with 10^7 *L. major* metacyclic promastigotes i.d. Three days before and 3 days after infection, they were treated i.d. with 250 μg of D ($n = 4$) or K ($n = 4$) ODNs at the site of challenge. Healthy monkeys treated with saline ($n = 6$) and SIV-infected monkeys that received control ODN ($n = 3$) or saline ($n = 3$) served as controls. Lesion size and SIV viremia were measured weekly. Three monkeys (one from control ODN and two from K-treated groups) were euthanized during the study due to weight loss and/or uncontrollable diarrhea. On day 56, the lesions were biopsied, the surviving animals were euthanized, and the local and systemic parasitic load was measured.

Parasite strains

L. amazonensis promastigotes (PH8) were grown in medium 199 supplemented with 20% FCS, 0.1 mM adenine (Life Technologies, Gaithersburg, MD), 25 mM HEPES (Life Technologies), 5 g/ml hemin (Sigma-Aldrich, St. Louis, MO), 1 g/ml biotin (Life Technologies), and Pen/Strep/L-glutamine (Life Technologies). *L. major* clone V1 promastigotes (MHOM/IL/80/Friedlin) were grown at 26°C in the same medium. Infective-stage metacyclic *L. major* promastigotes were isolated from 4- to 5-day-old stationary cultures by negative selection using peanut agglutinin (Vector Laboratories, Burlingame, CA), whereas infectious *L. amazonensis* promastigotes were purified by negative selection using mAb D5, as previously described (28, 30). In challenge experiments, 10^7 purified metacyclic promastigotes suspended in RPMI 1640 were injected i.d. into the forehead of macaques (15).

Parasite load

Parasite load was estimated as described (28). Briefly, 2-mm^2 biopsies were taken, treated with 1 mg/ml collagenase A (Sigma-Aldrich) for 2 h at 37°C, homogenized, filtered, and serially diluted in a 96-well flat-bottom microtiter plate containing biphasic medium, prepared using 50 μl of NNN medium containing 30% defibrinated rabbit blood and overlaid with 50 μl of M199/S. The number of viable parasites in each lesion was determined from the highest dilution at which promastigotes could be grown out after 7 days of incubation at 26°C. The total number of parasites in the lesion was obtained by multiplying the number of parasites in the biopsy by the area of the lesion.

Antibodies

Ab pairs that recognize both human and macaque IL-6 (R&D Systems, Minneapolis, MN), and IFN-α (PBL Biomedical Laboratories, New Brunswick, NJ) were used in ELISA. Abs specific for human (Endogen, Woburn, MA) or macaque (Bender MedSystems, Vienna, Austria; Mabtech, Stockholm, Sweden) were used to measure IFN-γ.

ELISA

Ninety-six-well microtiter plates (Millipore, Bedford, MA) were coated with anti-cytokine Ab and blocked with PBS-5% BSA (12). Culture supernatants were added, and their cytokine content was quantitated by the addition of biotin-labeled anti-cytokine Ab followed by phosphatase-conjugated avidin and phosphatase-specific colorimetric substrate. Standard curves were generated using known amounts of recombinant human cytokine. All assays were performed in triplicate. When supernatants from

Table I. *Characteristics of HIV-infected PBMC donors*

	<200 CD4$^+$ T Cells	200–500 CD4$^+$ T Cells	>500 CD4$^+$ T Cells
n	9	17	17
Age	40 ± 2	39 ± 1	37 ± 2
Race (white/black/Hispanic)	4/4/1	13/4/1	8/7/2
Gender (male/female)	8/0	15/2	17/0
CD4$^+$ T Cells	25 ± 7	317 ± 20	735 ± 67
% CD4$^+$ T Cells	3 ± 1	21 ± 1.9	31 ± 3
Viral load	27,000 ± 50,000	1,828 ± 29,000	663 ± 330
Viral load range	ND–75,000	ND–500,000	ND–35,000
% CD56$^+$/CD16 cells	9 ± 2	8.3 ± 1	5.6 ± 1.6
% CD19$^+$ cells	19.5 ± 5	14 ± 1	9 ± 2
% CD14$^+$ cells	19 ± 2	22 ± 1	15.6 ± 3
% on HAART	66	66	80

HIV/SIV-infected PBMCs were used, 0.02% Triton X-100 was added to the washing buffer to inactivate the virus.

Cell proliferation assay

A total of 10^5 PBMCs/well were incubated with 1–3 μM ODN for 68 h, pulsed with 1 μCi of [^{3}H]thymidine, and harvested 4 h later. All assays were performed in triplicate. Intraassay variation was <15%.

Flow cytometry

Cultured cells were washed in cold PBS, fixed, and stained with fluorescent-labeled Abs to CD4, CD56, CD16, CD19, B220, CD83, CD86, CD14, and MHC class II as previously described (18). Samples were washed and analyzed (20,000–40,000 events) on a FACScan flow cytometer (BD Biosciences, San Jose, CA). The number of DCs was obtained after gating on monocytes with proper electronic compensation. The data were analyzed with CellQuest software (BD Biosciences).

Viral load measurements

SIV plasma RNA levels were determined by a real-time RT-PCR assay, as described (31).

Statistical analysis

Statistically significant differences in cytokine and cell proliferation levels were determined using a two-tailed nonparametric rank sum test or ANOVA with Dunnett's post-test analysis. Spearmann's correlations were used to assess the relationship between viral load or number of CD4 T cells and response to ODNs. Differences in lesion sizes were tested by Friedman Repeated-Measures Analysis on Ranks with Tukey's All Pairwise Multiple Comparison Procedure using Sigma Stat (SPSS, San Rafael, CA). Differences in parasite load were tested by *t* test of log-normalized data.

Results

PBMCs from normal and HIV-infected donors respond to CpG ODNs

Retrovirus infection is associated with a progressive loss of immune function and increased susceptibility to opportunistic infections. CpG ODNs that activate PBMCs from normal human donors (14) were assessed for their ability to stimulate cells of the innate immune system of HIV patients. Consistent with previous reports, K ODNs preferentially induced cell proliferation and IL-6 production in PBMCs from healthy subjects, whereas D-type ODNs stimulated the secretion of IFN-α and IFN-γ (Fig. 1). As reported previously, pure phosphorothioate ODNs (non-CpG K-type controls) induced low-level, sequence-nonspecific cell proliferation. This phosphorothioate-dependent activation was significantly lower than that elicited by K ODN ($p < 0.05$) (5, 32). In addition, D ODNs, but not K ODNs, triggered the maturation of DCs in vitro, as characterized by increased expression of CD83 and CD86 (Fig. 2) (18).

PBMCs from HIV-infected and healthy subjects responded similarly to K-type ODNs (Fig. 1), suggesting that B cells and monocytes retained their ability to respond to this form of immune stimulation. Although D-type ODNs induced a significant increase in cytokine secretion by cells from both donor populations ($p < 0.001$), the IFN-α and IFN-γ response of healthy controls significantly exceeded that of HIV-infected subjects ($p < 0.05$ and $p < 0.001$, respectively; Fig. 1). This reduced responsiveness to D ODNs correlated directly with the number of CD4$^+$ T cells among the HIV-infected donors ($p < 0.01$; Fig. 3) and inversely with their viral load ($p < 0.05$; data not shown). No significant correlation between cytokine production and the number of CD56$^+$ NK cells or CD14$^+$ monocytes was observed (data not shown). D ODNs also maintained their ability to trigger the maturation of DCs from HIV-infected donors. As seen in the examples in Fig. 2, the absolute number of mature DCs was lower in the unstimulated PBMCs from HIV-infected donors than in normal donors (0.13 ± 0.06% vs 0.26 ± 0.07%, respectively; $p < 0.05$; data not shown). However, treatment with D ODNs increased the number of CD83$^+$CD86$^+$ cells by ~20-fold (to 2 ± 1% vs 6 ± 1%, respectively; data not shown) in both groups.

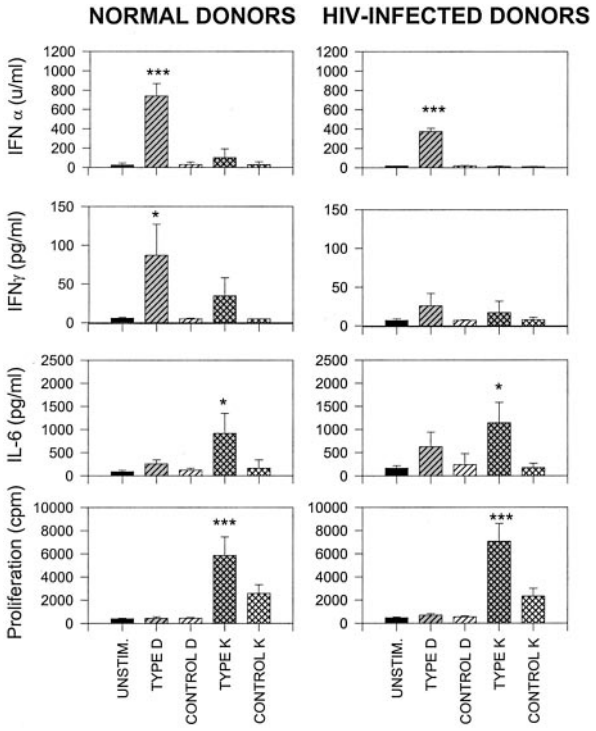

FIGURE 1. Response of human PBMCs to K and D ODNs. PBMCs from 16 healthy blood donors and 43 HIV-infected subjects were stimulated for 72 h with optimal concentrations of K3 (1 μM), D29 (3 μM), control K163 (1 μM), or control D122 (3 μM). IFN-α, IFN-γ, and IL-6 levels in culture supernatants were determined by ELISA, whereas cell proliferation was assessed by [^{3}H]thymidine uptake. Note that D ODNs induce the secretion of IFN-α and IFN-γ, whereas K ODNs induce higher cell proliferation and IL-6 production. All assays were performed in triplicate. Statistical significance was determined by ANOVA of log normalized data. *, $p < 0.05$; ***, $p < 0.001$.

PBMCs from normal and SIV-infected macaques respond to CpG ODNs

Rhesus macaques provide a useful model for evaluating the activity of CpG ODNs planned for human use (15, 16, 33, 34). Previous studies established that PBMCs from these animals respond to the same D and K ODNs that activate human PBMCs (15). We compared the responses of PBMCs from 16 immunocompromised SIV-infected animals to those of 20 healthy macaques. Consistent with results involving PBMCs from HIV-infected patients, PBMCs from SIV-infected macaques responded normally to K ODNs in vitro (Fig. 4). Their IFN-α response to D ODNs, by comparison, was significantly reduced when compared with PBMCs from healthy controls ($p < 0.01$; Fig. 4). Moreover, although healthy macaques responded to D ODNs by secreting IFN-γ, no detectable IFN-γ was detectable in PBMCs from SIV-infected macaques.

Immunoprotective activity of CpG ODNs in healthy macaques

Previous studies established that CpG ODNs can decrease the magnitude and duration of *Leishmania* infection in mice (8, 10, 35). A self-limiting cutaneous *L. amazonensis* challenge model was used to evaluate whether CpG ODNs could similarly protect rhesus macaques (26). Macaques were injected i.d. on days −3 and 3 with 500 μg of CpG ODNs that activate PBMCs from human

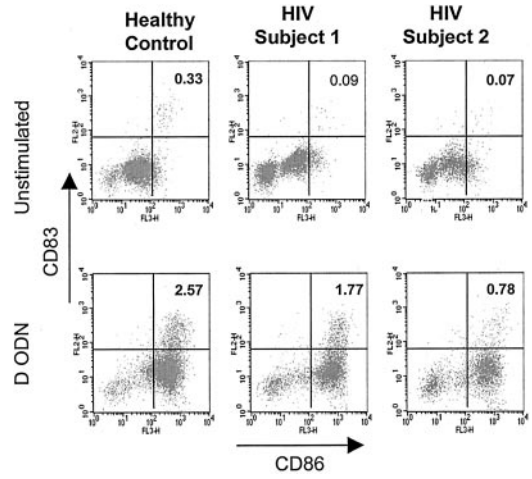

FIGURE 2. D ODNs induce monocytes to differentiate into mature DCs. Mature DCs ($CD83^+CD86^+$) were identified by FACS analysis of PBMCs from healthy and HIV-infected subjects. Note that the number of mature DCs in samples from HIV-infected patients increases 10- to 20-fold after 72 h of culture with 3 μM D ODN. Two representative examples of six experiments are shown.

and nonhuman primates (15). On day 0, the animals were challenged at the same site (forehead) with 10^7 metacyclic *L. amazonensis* promastigotes. Naive animals developed a cutaneous lesion similar to those found in human cutaneous leishmaniasis (26), with a peak surface area of 4.4 $\pm$ 0.7 mm^2 on day 22 (Fig. 5). Lesion size was significantly reduced among macaques treated with D-type ODN ($p < 0.001$; Fig. 5). In contrast, the severity of *Leishmania* infection in animals treated with K ODN was not significantly different from that of the controls ($p = 0.1$).

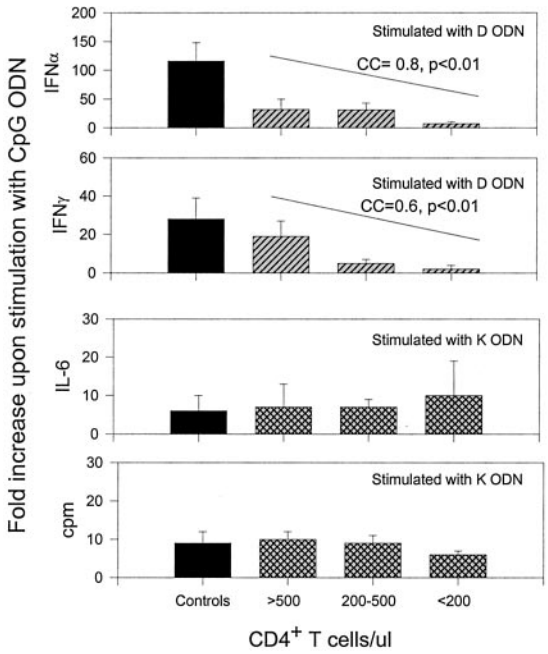

FIGURE 3. Relationship between $CD4^+$ T cell count and CpG ODN responsiveness. The response of PBMCs from HIV-infected donors ($n = 42$) to D (*upper two panels*) and K (*lower two panels*) ODNs was stratified by CD4 T cell count. Data show the fold increase in cytokine production and proliferation of treated vs unstimulated cells. The correlation coeficient (CC) and the p value were obtained using Spearman's Correlations.

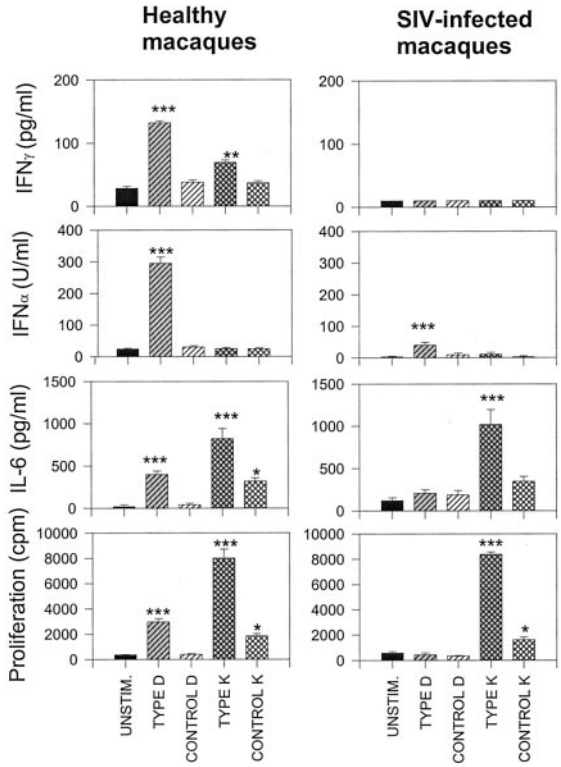

FIGURE 4. Response of PBMCs from SIV-infected and healthy rhesus macaques to CpG ODNs in vitro. PBMCs from 16 SIV-infected and 20 healthy macaques were stimulated for 72 h with K, D, or control ODN. IFN-γ, IFN-α, and IL-6 levels in culture supernatants were determined by ELISA, whereas cell proliferation was assessed by [^{3}H]thymidine uptake. The detection limit for the assays was 20 pg/ml for IFN-γ, IFN-α, and IL-6. All assays were performed in triplicate. Statistical significance was determined by a one-way ANOVA of log normalized data. *, $p < 0.05$; ***, $p < 0.001$.

Immunoprotective activity of CpG ODNs in SIV-infected macaques

Based on the observation that CpG ODNs retain the ability to activate PBMCs from retrovirus-infected primates, their ability to reduce the severity of a *Leishmania* infection in SIV-infected macaques was examined. Macaques that had been infected >12 mo earlier with SIVmac239 and that had viral loads ranging from 0.3 to 28 $\times$ 10^6 copies/ml were used in this study. The animals were stratified based on viral load and then were challenged with *L. major* metacyclic promastigotes (MHOM/IL/80/Friedlin). As shown previously, healthy macaques challenged with *L. major* developed cutaneous lesions characterized by erythema, induration, and ulceration that peaked 25 days after challenge and resolved within 50 days (Fig. 6*A* and Ref. 15). Due to their immunosuppressed state, the macaques developed severe progressive cutaneous lesions that did not resolve. The severity of *Leishmania* infection in animals treated with K ODNs was not significantly different from that of the controls. In contrast, macaques treated with D ODNs developed significantly smaller lesions, and their infection did not progress over time (Fig. 6*A*).

Animals were euthanized on day 56, and their parasite burden was measured. Monkeys treated with D ODNs had a 35-fold reduction in total parasite burden at the lesion site compared with SIV-infected animals treated with control ODNs or saline (Fig. 6*B*; $p < 0.001$). No systemic spread of the parasites was evident in any of the groups.

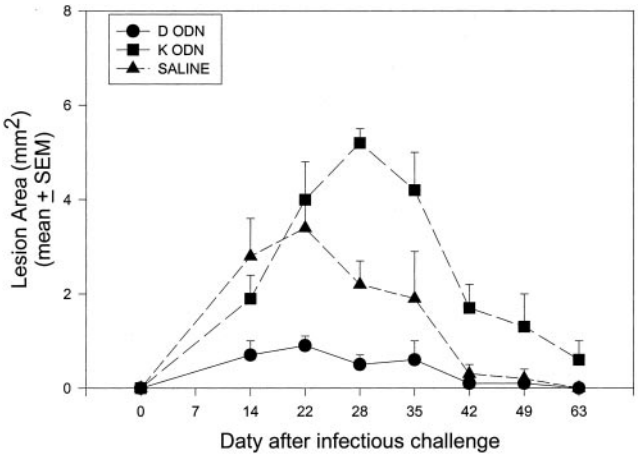

FIGURE 5. Effect of CpG ODN treatment on cutaneous leishmaniasis. Macaques (six per group) were treated with 500 μg of a mixture of D ODN, K ODN, or saline i.d. 3 days before and 3 days after an infectious challenge with 10^7 *L. amazonensis* metacyclic promastigotes. The mean and SEM of the area of the lesions is shown. Note that macaques treated with D ODN had significantly smaller lesions ($p < 0.05$).

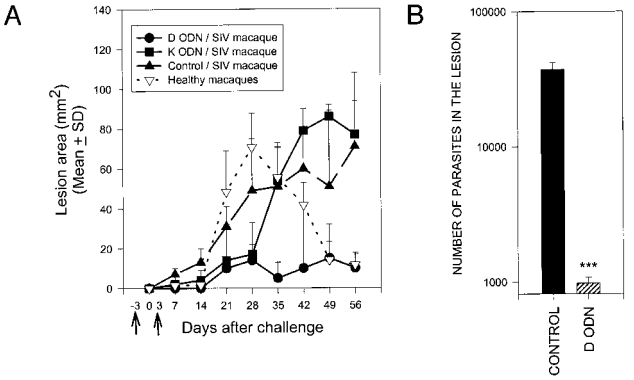

FIGURE 6. Effect of CpG ODN treatment on *Leishmania* lesions in SIV-infected monkeys. Macaques infected for >12 mo with SIVmac239 were treated with 250 μg of a mixture of D ($n = 4$) or K ($n = 4$) ODN i.d. 3 days before and 3 days after an infectious challenge with 10^7 *L. major* metacyclic promastigotes. Controls include untreated healthy macaques ($n = 6$) and SIV-infected macaques treated with either control ODN ($n = 3$) or saline ($n = 3$). *A*, Mean area of the lesions. *B*, Estimated total parasite load on day 56 in control vs D ODN-treated, SIV-infected macaques. Note that macaques treated with D ODN had significantly smaller lesions ($p < 0.05$) as well as lower parasite loads ($p < 0.001$).

Concurrent *Leishmania* infection can activate the HIV present in latently infected monocytes and T cells, thereby increasing viremia (36). Therefore, viral load measurements were conducted in *Leishmania*-infected macaques every 2 wk throughout the study. No significant change in viral load was evident in any of the groups (data not shown).

Discussion

CpG ODNs stimulate the innate immune system, thereby improving the host's resistance to infectious pathogens. Previous studies established that mice treated with CpG ODNs could survive otherwise lethal infections by *Listeria*, *Franciscella*, and *Leishmania* (8–11). Yet the CpG motifs that are highly active in rodents are poorly immunostimulatory in humans, limiting the utility of murine models to examine whether CpG ODNs can protect primates such as humans (12, 15). This study establishes that K and D CpG ODNs induce PBMCs from both normal and immunosuppressed primates to mature, proliferate, and secrete cytokines. Moreover, it demonstrates that CpG ODNs enhance the ability of both normal and immunosuppressed primates to resist pathogen challenge.

HIV infection results in not only a progressive reduction in $CD4^+$ T cells, but also a decrease in the number and functional activity of NK cells and plasmacytoid DCs as viral load rises (20, 21, 37, 38). Previous studies established that CpG ODNs activated PBMCs from normal donors; the present work extends that work to HIV-infected subjects. Although the number of mature DCs in the peripheral blood of HIV-infected donors is reduced (Fig. 2 and Refs. 21 and 39), PBMCs from retrovirus-infected humans and macaques responded to both D and K ODNs. Indeed, the magnitude of the response to K ODNs was essentially unaffected by retroviral infection, although the response to D ODNs was reduced in HIV- and SIV-infected donors. It is unlikely that the changes in responsiveness to D ODNs observed in PBMCs from HIV-infected donors were related to their antiretroviral therapy because 1) no significant correlation between the CpG response and antiretroviral therapy was evident and 2) a similar reduction in the response to D ODNs was evident in untreated SIV-infected monkeys. Despite the decline in IFN-γ and IFN-α response to D ODNs in SIV-infected macaques, the immune activation induced by these agents was sufficient to control a superinfection with *Leishmania*.

Murine studies established that protection against this parasite correlated with the production of type 1 cytokines, particularly IL-12 (40). It was unclear whether CpG ODNs could induce protection against *Leishmania* in primates because 1) primates and rodents respond optimally to different CpG motifs (12, 14) and 2) primates fail to produce large amounts of IL-12 when treated with CpG ODNs (41). Cutaneous infection of macaques with *Leishmania* provided a means for examining this question, because the nature, severity, and duration of this infection in macaques and humans is quite similar (26), and PBMCs from these species respond to the same CpG motifs (Figs. 1 and 4 and Refs. 15 and 28). As seen in Fig. 5, normal macaques treated with D ODNs developed significantly smaller lesions than control animals or animals treated with K ODNs after *L. amazonensis* infection. D ODN treatment of immunosuppressed SIV-infected monkeys also yielded protection against cutaneous leishmaniasis, despite their inability to induce IFN-γ, as reflected by smaller lesions and reduced parasite load (Fig. 6). Although viceral leishmaniasis rather than cutaneous leishmaniasis is of greatest concern in HIV patients, the reduced lesion size in these animals suggests that CpG treatment may contribute to the control of intracellular infections in these patients.

Because D ODNs excel at stimulating the production of Th1 cytokines and type 1 cytokines inhibit parasite proliferation, it is not surprising that D ODNs were the most effective at reducing the pathogenic effects of *Leishmania* infection (8, 42). In contrast, K ODNs neither stimulated Th1 cytokine production nor had any significant effect on the onset, magnitude, or duration of the *Leishmania* infection (8, 14, 17, 18). It is likely that functional differences between D- and K-type ODNs are due to differences in their structures. K ODNs have a phosphorothioate backbone and optimally contain multiple TCGTT and/or TCGTA motifs. D ODNs have a mixed phosphodiester/phosphorothioate backbone, contain a single self-complementary purine/pyrimidine/CpG/purine/pyrimidine motif, and are capped by a 3′ poly G tail (14). Ongoing studies suggest that these structural differences are associated with differences in the recognition, uptake, and/or processing of these two types of ODN by immune cells (43).

Over 30 million people are currently infected with HIV worldwide (44). Recent studies indicate that HIV patients are more susceptible to leishmaniasis (an estimated 9% of AIDS patients are co-infected with *Leishmania*) (45). In addition to the compromised immune status, HIV infection has been shown to enhance the intracellular growth of *Leishmania* in macrophages (46), which may explain why HIV patients tend to develop more aggressive visceral forms of that disease (45, 46). *Leishmanias*, in turn, can increase HIV viral load by activating latently infected monocytes and inducing chronic T cell activation (36). Current studies demonstrate that type D ODNs can reduce the severity of *Leishmania* infections by 35-fold in immunosuppressed subjects. The persistence of lesions even after D ODN treatment suggests that a combination of CpG ODNs with other antiparasitic agents may be required to cure this disease. Testing the efficacy of such combinations in both cutaneous and visceral models of leishmaniasis is an important goal of future research. Current results document that inducing a strong innate immune response can reduce host susceptibility to infection. Thus, CpG treatment may benefit normal individuals at increased risk of environmental exposure to infectious agents and may help to reduce the morbidity and mortality of opportunistic infections among the immunosuppressed. As such, CpG ODNs (alone or in combination with other agents) may become a valuable addition to conventional antiretroviral therapy.

Acknowledgments

We thank Drs. Joao Pedras-Vasconcellos and Sylvie Bartholet for reviewing the manuscript. We also thank Dr. Michael Piatak, Jr., for expert assistance with viral load analysis. In addition, we thank Dr. Phil Snoy, Ray Olsen, and the Animal Care Facility staff for their care of the nonhuman primates included in this study. Further thanks to Dr. Susan Leitman-Klinman and the personnel at the National Institutes of Health Department of Transfusion Medicine for providing human PBMCs. We thank Dr. Eduardo Romano for his assistance with the statistical analysis.

References

1. Medzhitov, R., and C. A. Janeway, Jr. 1997. Innate immunity: impact on the adaptive immune response. *Curr. Opin. Immunol. 9:4.*
2. Hemmi, H., O. Takeuchi, T. Kawai, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature 408:740.*
3. Kadowaki, N., S. Ho, S. Antonenko, R. W. Malefyt, R. A. Kastelein, F. Bazan, and Y. J. Liu. 2001. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *J. Exp. Med. 194: 863.*
4. Takeshita, F., C. A. Leifer, I. Gursel, K. Ishii, S. Takeshita, M. Gursel, and D. M. Klinman. 2001. Cutting edge: role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. *J. Immunol. 167:3555.*
5. Krieg, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol. 20:709.*
6. Klinman, D. M., F. Takeshita, I. Gursel, C. Leifer, K. J. Ishii, D. Verthelyi, and M. Gursel. 2002. CpG DNA: recognition by and activation of monocytes. *Microbes Infect. 4:897.*
7. Krieg, A. M., A. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature 374:546.*
8. Walker, P. S., T. Scharton-Kersten, A. M. Krieg, L. Love-Homan, E. D. Rowton, M. C. Udey, and J. C. Vogel. 1999. Immunostimulatory oligodeoxynucleotides promote protective immunity and provide systemic therapy for leishmaniasis via IL-12- and IFN-γ-dependent mechanisms. *Proc. Natl. Acad. Sci. USA 96:6970.*
9. Elkins, K. L., T. R. Rhinehart-Jones, S. Stibitz, J. S. Conover, and D. M. Klinman. 1999. Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. *J. Immunol. 162:2291.*
10. Zimmermann, S., O. Egeter, S. Hausmann, G. B. Lipford, M. Rocken, H. Wagner, and K. Heeg. 1998. CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J. Immunol. 160:3627.*
11. Krieg, A. M., L. L. Homan, A. K. Yi, and J. T. Harty. 1998. CpG DNA induces sustained IL-12 expression in vivo and resistance to *Listeria monocytogenes* challenge. *J. Immunol. 161:2428.*
12. Bauer, M., K. Heeg, H. Wagner, and G. B. Lipford. 1999. DNA activates human immune cells through a CpG sequence-dependent manner. *Immunology 97:699.*
13. Hartmann, G., and A. M. Krieg. 2000. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J. Immunol. 164:944.*
14. Verthelyi, D., K. J. Ishii, M. Gursel, F. Takeshita, and D. M. Klinman. 2001. Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. *J. Immunol. 166:2372.*
15. Verthelyi, D., R. T. Kenney, R. A. Seder, A. A. Gam, B. Friedag, and D. M. Klinman. 2002. CpG oligodeoxynucleotides as vaccine adjuvants in primates. *J. Immunol. 168:1659.*
16. Hartmann, G., R. D. Weeratna, Z. K. Ballas, P. Payette, S. Blackwell, I. Suparto, W. L. Rasmussen, M. Waldshmidt, D. Sajuthi, R. H. Purcell, H. L. Davis, and A. M. Krieg. 2000. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. *J. Immunol. 164: 1617.*
17. Krug, A., S. Rothenfusser, V. Hornung, B. Jahrsdorfer, S. Blackwell, Z. K. Ballas, S. Endres, A. M. Krieg, and G. Hartmann. 2001. Identification of CpG oligonucleotide sequences with high induction of IFNα/β in plasmacytoid dendritic cells. *Eur. J. Immunol. 31:2154.*
18. Gursel, M., D. Verthelyi, and D. M. Klinman. 2002. CpG oligodeoxynucleotides induce human monocytes to mature into functional dendritic cells. *Eur. J. Immunol. 32:2617.*
19. Pacanowski, J., S. Kahi, M. Baillet, P. Lebon, C. Deveau, C. Goujard, L. Meyer, E. Oksenhendler, M. Sinet, and A. Hosmalin. 2001. Reduced blood $CD123^+$ (lymphoid) and $CD11c^+$ (myeloid) dendritic cell numbers in primary HIV-1 infection. *Blood 98:3016.*
20. Azzoni, L., E. Papasavvas, J. Chehimi, J. R. Kostman, K. Mounzer, J. Ondercin, B. Perussia, and L. J. Montaner. 2002. Sustained impairment of IFN-γ secretion in suppressed HIV-infected patients despite mature NK cell recovery: evidence for a defective reconstitution of innate immunity. *J. Immunol. 168:5764.*
21. Chehimi, J., D. E. Campbell, L. Azzoni, D. Bacheller, E. Papasavvas, G. Jerandi, K. Mounzer, J. Kostman, G. Trinchieri, and L. J. Montaner. 2002. Persistent decreases in blood plasmacytoid dendritic cell number and function despite effective highly active antiretroviral therapy and increased blood myeloid dendritic cells in HIV-infected individuals. *J. Immunol. 168:4796.*
22. Howell, D. M., S. B. Feldman, P. Kloser, and P. Fitzgerald-Bocarsly. 1994. Decreased frequency of functional natural interferon-producing cells in peripheral blood of patients with the acquired immune deficiency syndrome. *Clin. Immunol. Immunopathol. 71:223.*
23. Corbett, E. L., R. W. Steketee, F. O. ter Kuile, A. S. Latif, A. Kamali, and R. J. Hayes. 2002. HIV-1/AIDS and the control of other infectious diseases in Africa. *Lancet 359:2177.*
24. Choi, C. M., and E. A. Lerner. 2002. Leishmaniasis: recognition and management with a focus on the immunocompromised patient. *Am. J. Clin. Dermatol. 3:91.*
25. World Health Organization. 2000. *Fact Sheet 116: Leishmania/HIV Co-infection in Southwestern Europe, 1990–1998*. World Health Organization, Geneva.
26. Amaral, V. F., V. A. O. Ransatto, F. Conceicao-Solva, E. Molinaro, V. Ferreira, S. G. Coutinho, D. McMahon-Pratt, and G. Grimaldi. 1996. *Leishmania amazonensis*: the Asian rhesus macaques (*Macaca mulata*) as an experimental model for the study of cutaneous leishmaniasis. *Exp. Parasitol. 82:34.*
27. Leifer, C. A., D. Verthelyi, and D. M. Klinman. Heterogeneity in the human response to immunostimulatory CpG oligodeoxynucleotides. *J. Immunother. In press.*
28. Kenney, R. T., D. L. Sacks, J. P. Sypek, L. Vilela, A. A. Gam, and K. Evans-Davies. 1999. Protective immunity using recombinant human IL-12 and alum a adjuvants in a primate model of cutaneous leishmaniasis. *J. Immunol. 163:4481.*
29. Lifson, J. D., M. Piatak, J. L. Rossio, J. Bess, E. Chertova, D. Schneider, R. Kiser, V. Coalter, B. Poore, R. Imming, et al. 2002. Whole inactivated SIV virion vaccines with functional envelope glycoproteins: safety, immunogenicity, and activity against intrarectal challenge. *J. Med. Primatol. 31:205.*
30. Campos-Neto, A., R. Porrozzi, K. Greeson, R. N. Coler, J. R. Webb, Y. A. Seiky, S. G. Reed, and G. Grimaldi, Jr. 2001. Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. *Infect. Immun. 69:4103.*
31. Lifson, J. D., J. L. Rossio, M. Piatak, Jr., T. Parks, L. Li, R. Kiser, V. Coalter, B. Fisher, B. M. Flynn, S. Czajak, et al. 2001. Role of $CD8^+$ lymphocytes in control of simian immunodeficiency virus infection and resistance to rechallenge after transient early antiretroviral treatment. *J. Virol. 75:10187.*
32. Zhao, Q., J. Temsamani, R. Z. Zhou, and S. Agrawal. 1997. Pattern and kinetics of cytokine production following administration of phosphorothiate oligonucleotides in mice. *Antisense Nucleic Acid Drug Dev. 7:495.*
33. Davis, H. L., I. I. Suparto, R. R. Weeratna, Jumintarto, D. D. Iskandriati, S. S. Chamzah, A. A. Ma'ruf, D. D. Nente, A. M. Krieg, Heriyanto, W. Smits, and D. D. Sajuthi. 2000. CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. *Vaccine 19:413.*
34. Jones, T. R., N. Obaldia, R. A. Gramzinski, Y. Charoenvit, N. Kolodny, S. Kitov, H. L. Davis, A. M. Krieg, and S. L. Hoffman. 1999. Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenic vaccine in Aotus monkeys. *Vaccine 17:3065.*
35. Walker, P. S., T. Scharton-Kersten, A. M. Krieg, L. Love-Homan, E. D. Rowton, M. C. Udey, and J. C. Vogel. 1999. Immunostimulatory oligodeoxynucleotides promote protective immunity and provide systemic therapy for leishmaniasis via IL-12 and IFNγ dependent mechanisms. *Proc. Natl. Acad. Sci. USA 96:6970.*
36. Wolday, D., N. Berhe, H. Akuffo, and S. Britton. 1999. Leishmania-HIV interaction: immunopathogenic mechanisms. *Parasitol. Today 15:182.*

37. Chehimi, J., S. E. Starr, I. Frank, A. D'Andrea, X. Ma, R. R. MacGregor, J. Sennelier, and G. Trinchieri. 1994. Impaired interleukin 12 production in human immunodeficiency virus-infected patients. *J. Exp. Med. 179:1361.*
38. Feldman, S., D. Stein, S. Amrute, T. Denny, Z. Garcia, P. Kloser, Y. Sun, N. Megjugorac, and P. Fitzgerald-Bocarsly. 2001. Decreased interferon-α production in HIV-infected patients correlates with numerical and functional deficiencies in circulating type 2 dendritic cell precursors. *Clin. Immunol. 101:201.*
39. Donaghy, H., A. Pozniak, B. Gazzard, N. Qazi, J. Gilmour, F. Gotch, and S. Patterson. 2001. Loss of blood $CD11c^+$ myeloid and $CD11c^-$ plasmacytoid dendritic cells in patients with HIV-1 infection correlates with HIV-1 RNA virus load. *Blood 98:2574.*
40. Gurunathan, S., D. M. Klinman, and R. A. Seder. 2000. DNA vaccines: immunology, application, and optimization. *Annu. Rev. Immunol. 18:927.*
41. Krug, A., A. Towarowski, S. Britsch, S. Rothenfusser, V. Hornung, R. Bals, T. Giese, H. Engelmann, S. Endres, A. M. Krieg, and G. Hartmann. 2001. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur. J. Immunol. 31:3026.*
42. Murphy, M. L., U. Wille, E. N. Villegas, C. A. Hunter, and J. P. Farrell. 2001. IL-10 mediates susceptibility to *Leishmania donovani* infection. *Eur. J. Immunol. 31:2848.*
43. Gursel, M., D. Verthelyi, I. Gursel, K. J. Ishii, and D. M. Klinman. 2002. Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. *J. Leukocyte Biol. 71:813.*
44. UNAIDS. 2002. AIDS epidemic update. Joint United Nations Program on HIV and AIDS, Geneva.
45. Morales, M. A., I. Cruz, J. M. Rubio, C. Chicharro, C. Canavate, F. Laguna, and J. Alvar. 2002. Relapses versus reinfections in patients coinfected with *Leishmania infantum* and human immunodeficiency virus type 1. *J. Infect. Dis. 185:1533.*
46. Wolday, D., H. Akuffo, G. Fessahaye, A. Valantine, and S. Britton. 1998. Live and killed human immunodeficiency virus type-1 increases the intracellular growth of *Leishmania donovani* in monocyte-derived cells. *Scand. J. Infect. Dis. 30:29.*