Clinical, Parasitologic, and Immunologic Evolution in Dogs Experimentally Infected with Sand Fly-Derived Leishmania chagasi Promastigotes

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Abstract. Experimental infection of dogs with Leishmania infantum has yielded heterogeneous clinical, parasitologic, and immunologic results. We studied dogs infected with 10^9 or 10^10 sand fly-derived promastigotes delivered by the intradermal (ID) or intravenous (IV) routes. Total mortality over 1 year post-infection reached 23.8%. The mortality and proportion of sustained polysymptomatic dogs was highest in the IV-10^10 group. The early appearance of polysymptoms was associated with an increased risk of progression to death. Dissemination of the parasite to lymph nodes was faster, and the subsequent infectivity to sand flies higher, in the IV compared with ID-infected dogs. Parasite-specific IgG1 or IgG2 production was similar among the groups, but higher interferon-γ (IFN-γ) and interleukin-10 (IL-10) expression was associated with polysymptomatic dogs. On the basis of the data obtained from this study, a sample size analysis using different endpoints for future vaccine trials is described.

INTRODUCTION

Visceral leishmaniasis caused by Leishmania infantum (= Leishmania chagasi) is a zoonotic disease with the potential to significantly impact children in rural and periurban areas of tropical and subtropical regions of the New World, and several countries of the Mediterranean basin.1 Canines have been the subject of multiple studies because their susceptibility to L. infantum makes them the most important domestic reservoir. The canine model also has a role in the study of the pathogenesis of visceral leishmaniasis, and for developing preclinical studies related to therapy and vaccination. Therefore, it is crucial to establish relevant, reproducible, and standardized infection protocols that could provide the framework for understanding mechanisms of pathogenesis, defining the immune response and surrogate markers associated with vaccine-induced protection, and identifying vaccine candidates against canine visceral leishmaniasis (CVL). The development of effective vaccines and anti-Leishmania drugs for preventing and treating CVL could serve two fundamental purposes by improving the health status of dogs and reducing their reservoir competence in endemic areas. Although some drug formulations such as liposomal antimony have shown some promise,2 the difficulty of treating symptomatic dogs with the standard antimonial therapy or second line drugs, underscores the need to develop a relevant infection model for preclinical trials.3-5

Experimental infections of dogs have been carried out using a wide range of strategies that included the use of cultured promastigotes,6-8 or tissue-derived amastigotes,6,9 Studies have used numbers of parasites that ranged from a few thousand to several million, delivered by the intradermal10-13 or intravenous routes.14-17 Some of these inoculations included an extract of Lutzomyia longipalpis salivary glands in an unsuccessful attempt to increase parasite pathogenicity.10,11 Notably, only on a few occasions were metacyclic promastigotes included in the inocula.10,11

In this study, we compared the intradermal and intravenous infection protocols in groups of mongrel dogs, using infective promastigotes of L. chagasi obtained from experimentally infected laboratory-reared Lu. longipalpis. Clinical, parasitologic, immunologic, and pathologic parameters were assessed to determine the advantages and disadvantages of each infection protocol.

MATERIALS AND METHODS

Animals. Mixed-breed female puppies, 4–5 months of age, were obtained from the Centro de Zoonosis of Cali or purchased from private owners. All dogs had resided only within areas non-endemic for visceral leishmaniasis. The animals were handled and housed following international and Colombian guidelines (Law 84/89), in CIDEIM’s kennel near Cali where neither Leishmania transmission nor sand fly activity has been recorded. The research protocols were approved by the animal care and use committee at CIDEIM and the UTHSCSA Institutional Animal Care and Use Committee. Before the experimental infection the animals were quarantined under observation for 2 weeks, subjected to treatment of common intestinal parasites (Triantelm, Intervet; Rondel, Virbac Laboratories; Ivomec, Merial) and vaccinated against frequent dog pathogens (Galaxy DH2PPiL, Wyeth-Fort Dodge Laboratories; Novicac Rabia, Intervet). Dogs were negative for anti-leishmanial antibody by enzyme-linked immunosorbent assay (ELISA) (see method below).

Parasites and parasite antigens. The Leishmania chagasi strain (MCAN/COL/98/CATIRE) used for all experimental infections was isolated previously from a dog with CVL that inhabited the endemic focus of the municipality of Nilo (4°18’N, 74°42’W), Cundinamarca, Colombia. Leishmania chagasi promastigotes were routinely cultured in Schneider’s culture medium. To obtain amastigotes for infecting sand flies, hamsters were inoculated intravenously with 10^7 L. chagasi promastigotes and killed 3 mo p.i. Amastigotes were harvested from the spleen, washed twice in phosphate buffered saline (PBS) (at room temperature), and mixed with inactivated (56°C, 30 min), defibrinated rabbit blood at a final concentration of 3 x 10^6 amastigotes per mL. The parasite-laden blood was placed in a plastic container covered with a fresh chick skin

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membrane through which colonized Lu. longipalpis were allowed to feed. Engorged flies were maintained on a sugar-water diet at 26°C and dissected 11–12 days post-feeding in a drop of sterile PBS under a dissecting microscope. At this point in time the majority of isolated promastigotes had the morphology typical of metacyclic, infective forms. The head and anterior part of the gut (mainly the stomodeal valve) of 10 to 15 flies were teased apart and pooled in RPMI medium with 10% FCS. The suspension of infected sand fly tissue was agitated by up and down pipetting and the number of parasites quantified approximately every 15 min using a Neubauer chamber. Sand fly-infected tissues were added to the suspension as needed until the number of promastigotes required to infect 2–4 dogs was reached. Within 1 hr of parasite harvesting, the dogs were inoculated intradermally (ID) in the ear or intravenously (IV) with either 104 or 105 L. chagasi promastigotes.

**Clinical assessment.** Animals were examined on a weekly basis looking for signs and symptoms of visceral leishmaniasis, which included weight loss, enlarged lymph nodes, hepatosplenomegaly, keratoconjunctivitis, onychogryphosis, alopecia, dermatitis, skin ulcers, secondary bacterial infection, anemia, and coagulopathy evidenced by bleeding through natural orifices.

**Parasitologic evaluation and infectivity for vectors.** Every 2 months, popliteal lymph node aspirates were seeded in Schneider’s culture medium supplemented with 20% FCS and incubated at 25°C with regular inspections in an inverted microscope every 5 days for up to 3 weeks. The capacity of dogs to transmit L. chagasi was determined by means of xenodiagnosis, carried out with laboratory colonized Lu. longipalpis originally collected in Nilo, Cundinamarca. Sand flies were transferred to 250 mL plastic containers covered with fine mesh, and were deprived of sugar 24 hr before xenodiagnosis. The dogs were anesthetized with xylacine (2 mg/kg; Rompun, Bayer, Colombia) and 40–50 sand flies were allowed to feed for 30 min through the fine mesh of the container, which was pressed against the internal surface of the ears. Between 5 and 6 days post-feeding, flies were individually dissected under a dissecting microscope searching for promastigotes in the digestive tract. The proportion of infected flies and the intensity of infection were recorded as described by Travi and others.18

**Parasite-specific antibody determinations.** Anti-L. chagasi antibody levels were determined at different intervals by ELISA performed in 96-well microplates (Inmunolon 2, Dynex Technologies, Chantilly, VA) using 1 μg of soluble L. chagasi antigen (SLCA) per well, 50 μL of peroxidase-labeled rabbit anti-dog IgG (H+L) HRP (ICN Biomedicals, Irvine, CA) diluted at 1:5,000. The same methodology was applied to determine IgG1 and IgG2 levels, using affinity purified, peroxidase-labeled polyclonal antibodies developed for these subclasses (Bethyl Laboratories, Montgomery, TX) diluted at 1:8,000 in 1% BSA. Animals were considered seropositive when the optical density of the serum was > 3 standard deviations above the mean of six normal, uninfected controls.

**Culture of PBMCs for lymphoproliferation and cytokine analysis.** Canine peripheral blood mononuclear leukocytes (PBMCs) were isolated from heparinized blood by Ficoll-Hypaque gradient (Histopaque-1119, Histopaque-1077, Sigma, St. Louis, MO) and suspended in RPMI with 10% FCS and 1.74 μL (50 μM) β-mercaptoethanol 2 × 108 cells in 200 μL of medium per well were stimulated either with Con A (0.4 mg/mL for 4 days) or 25,000 (by freeze-thawing) killed L. chagasi promastigotes for 6 days at 37°C in a 5% CO2 atmosphere. Cells were pulsed for the final 8 hr of culture with 1 μCi of 3H-methyl-thymidine and counted in a scintillation counter. Results were expressed as stimulation index (net counts per min of stimulated cells/net counts per min of unstimulated cells). For cytokine analyses, PBMCs from control and infected dogs were cultured at 37°C, 5% CO2 in 2 mL round-bottomed polypropylene tubes with medium alone or in the presence of 2.5 μg/mL of Con A (Sigma), 25 μg/mL SLCA, or 4 × 106/mL frozen-thawed L. chagasi promastigotes for 120 hr. After stimulation, the supernatant was harvested and maintained at −20°C for subsequent determination of interleukin-10 (IL-10) and interferon-γ (IFN-γ) production by ELISA as recommended by the supplier (Quantikine, Canine IL-10; DuoSet, canine IFN-γ, R&D Systems, Minneapolis, MN). The cell pellets were then washed by centrifugation in cold PBS and suspended in 180 μL of RNAlater (Ambion Inc., Austin, TX) for RNA preservation. The cells were stored at −70°C until the RNA extraction was performed.

**RNA isolation.** The cell pellet preserved in RNAlater was diluted with 10 volumes of RNA lysis buffer (guanidinium based) and the RNA was isolated according to the manufacturer’s protocol (QIAGEN Inc., Valencia, CA). Total RNA was eluted from the binding column in 50 μL of nuclease-free water. To enhance the susceptibility of any contaminating DNA to DNase digestion, the RNA was heated for 3 min at 95°C, quenched on ice, and then treated with 50 units of RNase-free DNase (Stratagene, La Jolla, CA) for 1 hr at 37°C. The RNA extraction was then repeated and the RNA eluted in 35 μL of nuclease free water and stored at −70°C until used.

**Determination of cytokine expression by RT-real time-PCR.** The primers and probes used in this study and their optimization has been described previously.19,20 Each sample was assayed for cytokine expression and for 18S ribosomal RNA (rRNA) as an internal control in the same tube in a 25 μL reverse transcription-polymerase chain reaction (RT-PCR) mixture. The mixture contained a final concentration of 100 nM primers and probe (IFN-γ); 300 nM primer and 100 nM probe (IL-4 or IL-10), and 50 nM primer and probe (18S rRNA), 50 μL of 2X master mix, 2.5 μL of 40X multiprime (both in TaqMan One-Step RT-PCR Master Mix Reagents, Applied Biosystems, Foster City, CA) and 10 ng of template RNA. The samples were placed in MicroAmp, optical tubes, (Applied Biosystems) and amplified in an automated fluorometer (ABI Prism 7700 Sequence Detection system; Applied Biosystems). Amplification conditions were 48°C/30 min (Reverse Transcription), 95°C/10 min (AmpliTaq Gold Activation), 95°C/15 sec (PCR, denature), and 60°C/1 min (PCR anneal/extend). The spectral compensation for the post run analysis was made to improve dye spectral resolution. The relative quantity of cytokine messenger RNA (mRNA) was determined using the Comparative ΔACT method.21 This method enabled relative quantification of template and increases sample throughput by eliminating the need for standard curves. The values were expressed relative to a reference sample (calibrator), which in our experiments was unstimulated PBMCs. The Ct for the target and the Ct for the internal control were determined for each sample.

**Pathologic evaluation.** At the end of the study period (12 mo p.i.) the dogs were killed with Euthanex and the macroscopic appearance of the liver and spleen was examined.
Samples of these organs and lymph nodes were preserved in buffered formalin and 5 μm thick tissue sections stained with hematoxylin-eosin were evaluated by light microscopy with the observer blinded to the sample group.

**Statistical analyses.** Differences between groups were determined by analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis test. The Duncan Test was run for multiple comparisons after the ANOVA test. Differences in proportions were evaluated by Fisher’s exact test. Differences in cytokine expression were also determined using the Mann-Whitney test, except for some time points involving dogs infected intravenously with 10⁵ promastigotes caused by the low number of samples available (N < 3). All analyses were performed at 0.05 significance level using SPSS 7.5 standard version for Windows (SPSS, Inc., Chicago, IL) or InStat (GraphPad) version 3.0 for Windows.

**RESULTS**

**Clinical evolution.** The clinical evolution of *L. chagasi* infection in four different groups of dogs was determined over the course of 12 months. Dogs (N = 4–6 per group) were inoculated ID in the ear or IV with either 10⁴ or 10⁵ *L. chagasi* promastigotes that had been isolated from infected sand flies, and the animals were examined weekly. The frequency of signs and symptoms of visceral leishmaniasis (VL) fluctuated throughout the observation period in all the groups, with signs and symptoms appearing and regressing at different times post-infection. There was no significant difference in the frequency of the individual signs and symptoms among the four different inoculation groups. When all of the infected dogs were considered together, the most common VL signs were polyadenopathy, (86%) fur changes such as a dull coat, brittle hair, or partial alopecia (57%), cachexia (48%), onychogryphosis (31%), dermatitis or cutaneous ulceration (20%), and lethargy (20%). Although the limited number of dogs in some of the groups hindered the statistical analysis, it was observed that the highest proportion of sustained polysymptomatic individuals was found in dogs infected with 10⁵ promastigotes through the IV route (Figure 1). When the prevalence of polysymptoms (number of polysymptomatic dog-months) was compared, the large-dose (10⁵) IV group had a higher proportion of polysymptomatic dog-months than the group that received 10⁴ parasites by the IV route (20/48 versus 12/72, respectively; P = 0.0032) or the group that received 10⁵ parasites by the ID route (20/48 versus 7/60, respectively; P = 0.0006). Throughout the study period the prevalence of polysymptoms in the ID4 group was low except for an unexplained sharp increase at 6 mo p.i. Because of this transient peak the cumulative frequency of polysymptoms in this group was not statistically different from that of the large-dose IV group (P = 0.33). The early appearance of polysymptoms was associated with an increased risk of progression to severe disease or death since 4/5 dogs that died of VL developed symptoms before the 6 mo p.i. as compared with 3/13 that were either asymptomatic or oligosymptomatic until the 12 mo p.i. (P = 0.0251). By the 12 mo p.i. all of the surviving dogs were either oligosymptomatic (8/16) or asymptomatic (8/16) indicating that they had controlled *L. chagasi* infection. Half of the dogs that at the 12 mo p.i. were oligosymptomatic had been polysymptomatic at some point in time, but only one of the eight asymptomatic dogs had been previously polysymptomatic (P = 0.040).

Total mortality over the 1-year course of infection was 23.8%. The highest mortality was observed in dogs infected with 10⁵ promastigotes through the IV route. In this group, 2 of 4 dogs died at the 5 and 8 mo p.i. Each of the other groups had one death (ID-10, N = 6; IV-10, N = 6; ID-10, N = 5), and the earliest death (2 mo p.i.) occurred in a dog infected IV with 10⁴ promastigotes.

We reasoned that the level of anemia in the infected dogs might be an additional marker of disease severity. The lowest hematocrit values in all groups were recorded in the 6 mo p.i. (Figure 2), and polysymptomatic dogs, regardless of the infection protocol, had significantly lower values at this time point than did oligosymptomatic dogs (P = 0.030), asymptomatic dogs (P = 0.013), or uninfected controls (P = 0.0025). At other time points, there was no clear difference in hematocrit values between clinical groups or experimental infection protocols (Figure 2). However, there was a significant difference in hematocrit nadirs (mean ± SD) between the dogs that died of VL (26.6, ± 5) and those that survived the infection (32.8, ± 7.2) (P = 0.043).

**Parasitologic evolution.** Aspirate-culture of popliteal lymph node (distant from the site of inoculation) was used as an indicator of infection evolution. None of the dogs were parasitologically positive at 2 mo p.i. When each of the four groups was considered individually, there was no significant difference in the rates of parasite detection. Comparison of the data from the dogs that were infected intravenously or intradermally, showed that *L. chagasi* disseminated earlier in the IV infected group than in the ID infected animals, as shown by the higher proportion of parasitologically positive individuals at 4 mo p.i. (IV: 5/9 versus ID: 1/11, P = 0.049; Figure 3). As infection progressed, an increasing number of ID infected dogs had positive poplytal lymph nodes and consequently the difference between the two routes of inoculation became less evident (IV: 7/9 versus ID: 4/11, P = 0.080). Grouping of dogs according to the size of the inoculum showed that the number of infecting promastigotes had no impact on the proportion of animals with detectable parasites in the lymph nodes (10⁴ promastigotes: 8/12 versus 10⁵ promastigotes: 4/9, P = 0.198). There was a greater tendency for dogs that were polysymptomatic at some time during the year of observation to have a positive lymph node (LN) culture
compared with the group of dogs that were asymptomatic or oligosymptomatic ($P = 0.07$).

**Immune response.** We wanted to determine if the route of delivery or quantity of parasites delivered would influence the subsequent parasite-induced humoral and cellular immune responses. Furthermore, because previous studies of naturally infected dogs showed strong cellular immune responses in the dogs that did not progress to full blown VL, but weak cellular and strong antibody responses in those with progressive disease, we wanted to compare the responses of symptomatic and asymptomatic dogs. An antigen-specific IgG response following infection with *L. chagasi* was detected at the 2 mo p.i. in all groups except the low-dose IV challenge group (Figure 4). From that time forward, the IgG response increased in all groups, reaching a maximum at 4–6 mo p.i. and then either remaining stable or decreasing. There was considerable dog to dog variation. In general, over the course of observation, intradermal infections produced lower antigen-specific IgG levels than intravenous infections. At 6 mo p.i. the antibody production was slightly higher in intravenously infected dogs than in intradermally infected dogs (mean O.D. 1.0 ± 0.6 versus 0.6 ± 0.3: $P = 0.0568$), but this difference between routes of inoculation diminished with time of evolution becoming not significant at the end of the study period ($P = 0.197$) (Figure 4). However, at 12 mo p.i. antibody production was associated with symptoms, i.e., oligosymptomatic dogs had higher antibody levels (mean O.D. 0.92 ± 0.49) than asymptomatic individuals (mean O.D. 0.50 ± 0.15) ($P = 0.034$).

Intravenously infected animals showed a modest but steady increase in IgG1 that was significantly greater than uninfected controls at 8 mo p.i. ($P = 0.02$; data not shown). In contrast, intradermally infected dogs had a non-significant increment in IgG1 levels at 6 mo p.i., declining thereafter ($P = 0.304$; data not shown). The IgG2 levels were moderately elevated during the early stages of infection in all the animals and tended to increase with time of evolution in both ID and IV infected animals with no obvious differences between the groups (data not shown). When symptomatic or asymptomatic dogs were compared (Figure 5), no differences in IgG1 or IgG2 production were observed at any of the time points. The level of IgG2 increased significantly in symptomatic dogs from the 4th to the 8th ($P = 0.0255$) and 12th ($P = 0.0002$) mo p.i. and in asymptomatic dogs from the 4th to the 12th mo p.i. ($P = 0.0184$) (Figure 5). In contrast, no increase in IgG1 levels was observed in infected compared with uninfected dogs (Figure 5).

Lymphocyte proliferation in response to *L. chagasi* showed considerable dog to dog variability, and was generally low and similar in all infection protocols, even when dogs were grouped as symptomatic or asymptomatic (data not shown). There was a modest increase in lymphoproliferation following the 6 and 12 mo p.i. in the group infected IV with $10^5$ promastigotes, but this observation was based only on two surviving dogs (Figure 6). Thus, these data suggested that dogs that survived the largest inoculum developed a good cellular immune response to the parasite. All the infected dogs responded to the mitogen Con A similarly to the control group, and no differences between experimental groups were found throughout the study period (data not shown).

Antigen-induced IFN-γ and IL-4 mRNA expression was always low or undetectable in PBMCs from uninfected dogs, but high-level expression (30–121-fold increase compared with unstimulated cells) was always induced by the non-specific mitogen Concanavalin A in both control and infected dogs (data not shown). The highest antigen-induced IFN-γ expression occurred consistently at 6 mo p.i. in all experimental groups, as determined by *in vitro* PBMC recall stimulation with SLCA (Figure 7). High IFN-γ mRNA expression

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**Figure 2.** Evolution of hematocrit in dogs infected through the intradermal (ID) or intravenous (IV) routes with $10^4$ (4) or $10^5$ (5) metacyclic promastigotes of *Leishmania chagasi* harvested from experimentally infected *Lutzomyia longipalpis*. The lowest hematocrit values were found at the 6 mo p.i. in all the groups regardless of the infection protocol. (The data are expressed as mean, SD.)

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**Figure 3.** Parasitologic evolution of dogs experimentally infected through the intradermal (ID) or intravenous (IV) routes with $10^4$ (4) or $10^5$ (5) metacyclic promastigotes of *Leishmania chagasi* harvested from experimentally infected *Lutzomyia longipalpis*. The proportion of positive individuals was established by means of aspirate-culture of popliteal lymph nodes.
was associated with polysymptomatic individuals (mean ± SD relative mRNA expression = 106 ± 107) as opposed to asymptomatic/oligosymptomatic animals (18 ± 23.6), irrespective of the infection scheme \((P = 0.0365)\). The results obtained by ELISA at 2 and 6 mo p.i., although not statistically significant, showed that production of IFN-γ by PBMCs stimulated with \(L. chagasi\) antigen was more frequently detected and tended to be higher in symptomatic compared with asymptomatic dogs (Table 1). No statistical differences in IL-10 production by PBMCs were found when symptomatic and asymptomatic dogs were compared. However, in the symptomatic group, a trend of growing IL-10 production and a significant increasing frequency \((P = 0.0152)\) of animals producing this cytokine was observed between the early and late stages of infection. However, only half of the asymptomatic dogs produced low levels of IL-10 throughout the infection course (Table 1). Discrimination of cytokine production by route of inoculation (IV or ID) indicated that IL-10 was produced earlier by the majority of intravenously infected dogs \((P = 0.007; Table 2)\). Conversely, this inoculation route elicited a negligible IFN-γ production throughout the observation period (Table 2).

**Infectivity to sand flies.** We determined the capacity of the infected dogs to transmit the parasite to the sand fly vector during a bloodmeal. The sand flies were laboratory reared to exclude the possibility of natural infections. Between the 4th and 6th mo p.i., 3 dogs (all inoculated by the IV route) were infective to sand flies. Thus, dogs inoculated by the IV route tended to have a higher rate of infectivity to the vector \((3/9)\), compared with dogs that had been inoculated through the ID route \((0/11)\) \((P = 0.07)\). With the exception of a single moribund dog (subjected to xenodiagnosis just before euthanasia), in which the sand fly infection rate reached 79%, infectivity in general was transient and of low intensity (3–4% of flies infected), and coincided with the time point when dogs were oligosymptomatic \((N = 1)\) or progressing to a polysymptomatic condition \((N = 2)\).

**Pathologic findings.** Polyadenopathy was a common clinical sign in both ID and IV infected dogs. The spleen was found enlarged, with increased granularity, apparently at the expense of the white pulp, in a small proportion of dogs (ID: 3/8, IV: 1/5) that were necropsied at the end of the study. No macroscopic liver alterations were observed. In general, the histopathologic picture showed an inflammatory process of the liver and spleen, which was consistently more severe in intravenously infected animals \((4/5)\) than in intradermally infected dogs \((0/8)\) \((P = 0.007)\). The spleens showed areas of acute or chronic inflammation, lymphoid hyperplasia, and accumulation of epithelioid histiocytes. The liver had a mixed inflammatory infiltrate, predominantly chronic, in the peri-central and portal areas, including the lobules in some individuals. Most of the dogs showed chronic polylymphadenitis with follicular hyperplasia, sinusoidal histiocytosis and plasma cell infiltration, Russel bodies and accumulation of neutrophils. Small liver granulomas were observed principally in intradermally infected dogs.

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**Figure 4.** IgG antibody titers determined by enzyme-linked immunosorbent assay (ELISA) in dogs experimentally infected with metacyclic promastigotes of *Leishmania chagasi*. (Data expressed as mean, standard deviation; dotted line indicates cut-off point established at 3 SD above uninfected controls).

**Figure 5.** IgG1 or IgG2 subclass antibody titers determined by enzyme-linked immunosorbent assay (ELISA) in symptomatic or asymptomatic dogs experimentally infected with *Leishmania chagasi*. Oligosymptomatic and polysymptomatic dogs were all grouped as symptomatic dogs. The dotted line represents the O.D. of pooled sera of uninfected dogs. Data are expressed as mean and standard deviation. The dotted line represents the optical density of uninfected dogs.
DISCUSSION

The domestic dog is the primary natural reservoir host of Leishmania infantum/chagasi, and as such, is an important target for vaccination and treatment to reduce the burden of canine disease and interrupt transmission to the human population. For vaccine and treatment studies to proceed in an efficient manner, a clinico-epidemiologically relevant experimental infection model is needed. In this study we describe the clinical, parasitologic, and immunologic outcomes in dogs infected with sand fly–derived promastigotes. We compared intradermal infection, mimicking the natural route of exposure, to intravenous infection, which has been the approach used for the majority of studies of experimental canine VL.

In our study, both intradermal and intravenous infections led to the development of symptomatic VL and fatal infections in some dogs within 2–6 mo p.i. The mortality rate was low regardless of the infection route, but dogs infected by the intravenous route developed a higher prevalence of symptoms of VL. However, the majority of dogs that survived were asymptomatic at 1-year p.i., which is similar to what has been observed in endemic areas of the Mediterranean basin, where a large proportion of L. chagasi–infected dogs remain asymptomatic or develop only transient disease after infection. In contrast, in Colombia we have seen a higher frequency of overt canine VL, which is frequently associated with internal and external poly-parasitism and malnourishment.

Even under the controlled conditions of this study, where the dogs were housed together, received the same diet, and were eradicated of intestinal parasites before L. chagasi infection, the clinical outcome ranged from asymptomatic infection to death. Studies of naturally infected dogs also suggest that factors inherent to the host contribute to the variability in outcome of infection. In fact, most infection protocols in vaccination or treatment studies appear to be designed to overcome the natural resistance of many dogs and produce full-blown disease in a short time span by delivering a massive dose of parasites by the unnatural route of IV injection.

Previous studies showed that experimental IV infection was more likely to lead to overt disease than intradermal inoculation, which typically led to subclinical infection. Our finding of small liver granulomas, an indication of an active cell mediated immune response, in the intradermally infected dogs is consistent with what was observed in resistant dogs naturally infected in endemic areas. However, the previous work by Killick-Kendrick and others showed development of severe disease (timing or nature not specified) in 48% of dogs infected intradermally with approximately 10^5 L. infantum promastigotes isolated from the mid guts of infected sand flies. These dogs were followed for a prolonged period (5 years), whereas the dogs in our study were followed only for 1 year. Thus, it is possible that longer periods of obser-
expression could unmask progression to overt VL in dogs that after 1 year are still oligosymptomatic.

The IFN-γ is well established to be a key mediator of resistance of macrophages to *Leishmania* infection. A large number of studies using putatively protective antigens or attenuated parasites in mice have shown that protection against progressive visceral infection involves high expression of IFN-γ and decreased expression of IL-10.32–34 In dogs, low parasite burdens of *L. chagasi* in lymph nodes also were associated with high expression of IFN-γ and TNF-α.35 In this study, expression and production of IFN-γ by PBMCs was detected earlier in the intradermally infected dogs than in those infected by the intravenous route, but the peak in parasite-induced IFN-γ occurred at 6 mo p.i., regardless of the route of infection. Delayed cytokine production was shown previously in experimentally infected dogs4 and mice36 and has been described as a phase of silent establishment and dissemination of *Leishmania* in the absence of pathology. Most significantly in our study, 67% of symptomatic dogs (regardless of the infection protocol) produced high levels of IFN-γ at the early stages of infection, and the proportion of individuals producing this cytokine increased over time, indicating that IFN-γ production was not sufficient to prevent disease, or was it a good surrogate marker of resistance. The elevated expression of IFN-γ during severe disease has also been described in patients with active VL.36,37

In contrast to IFN-γ, parasite-induced production of IL-10, a macrophage deactivating cytokine, was evident early in the IV but not ID infected dogs. Over time there was a significant increase in the proportion of dogs producing IL-10, and the symptomatic dogs tended to produce more IL-10 than the asymptomatic dogs, consistent with the findings of elevated IL-10 mRNA expression in lymph nodes of dogs with active VL,35 and the increased IL-10 in humans with active VL.36,37 Although the observed variability in IFN-γ and IL-10 production hinders their use as predictors of disease evolution at the individual level, analyses of these parameters in large groups involved in vaccine trials could be informative.

As described in other studies,38–40 all the experimentally infected dogs in our study developed anti-*L. chagasi* antibody responses. However, in contrast to some studies of naturally infected dogs41,42,43,44 there was no correlation between clinical symptoms and antibody titer. Several previous studies have associated high IgG2 production with asymptomatic infections, and elevated IgG1 levels with disease.42–44 Our results and the findings of others45,46 did not identify a clear association of symptomatic disease with a particular isotype reactivity. We did not measure parasite-specific IgE, which may be more effective than the IgG2/IgG1 ratio in identifying disease progression in challenge studies.46

The efficiency of vector-mediated transmission of *L. chagasi* in the canine population of endemic areas has not been clearly defined. In this study, and in previous studies in Latin America,18,47,48 the majority of infected dogs were non-infective to laboratory-reared sand flies. Our finding that only symptomatic dogs infected by the IV route were infective to sand flies is consistent with previous observations that the proportion of flies that acquire the infection on a given dog correlates with the severity of the disease.18,48,49 In contrast to these observations in Latin America, both asymptomatic and poly-symptomatic dogs from the Mediterranean basin were shown to be highly infective to sand flies.50 This could be caused by the marked vector competence of the Old World species *Phlebotomus perniciosus* as opposed to the New World species *L. longipalpis*.18 Therefore, it could be speculated that immunoprophylactic approaches that prevent animals from reaching the polysymptomatic stage could have greater impact.

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<tr>
<td>12</td>
<td>Median (range) (pg/mL)</td>
<td>96.9 (291)</td>
</tr>
<tr>
<td></td>
<td>Proportion* (%)</td>
<td>6/7 (86)</td>
</tr>
</tbody>
</table>

*Number of dogs producing interleukin-10 (IL-10) or interferon-γ (IFN-γ)/total dogs in the group.
†Significant difference between 2 and 12 mo p.i. within the symptomatic dogs, as determined by Fisher’s exact test *P* = 0.015.
in controlling transmission in the Americas than in the Old World.

We believe that if a more relevant (smaller dose, intradermal inoculation) challenge model in vaccine trials is used, then endpoints other than death, such as morbidity (symptomatic months), infectivity to sand flies, and parasite burden, must be used, and the vaccine trial must include a large number of animals. Using data from the current study, and making the assumption that parasites in a prospective challenge would have virulence similar to those used in this study, and an α (type I error) value of 0.05 and a power (1-β) of 0.90, the calculated sample sizes necessary to effectively show a vaccine-induced reduction in several endpoints are shown in Table 3. This analysis indicates that many of the previous canine vaccine trials were probably underpowered because of the small sample size. Furthermore, if the ID challenge model is used, then it is obvious that a larger number of animals per group would be required compared with those necessary in the IV challenge model.

In summary, this study of dogs infected intradermally or intravenously with a relatively small number of sand fly-derived promastigotes provides a number of insights into the disease evolution, transmission, and the potential for interruption of canine VL. First, the pattern of disease evolution in the infected dogs was dynamic, with signs and symptoms appearing and regressing at different times post-infection. A standardized inoculum, whether administered by the intradermal or intravenous route, led to the development of a spectrum of disease ranging from subclinical infection to rapidly fatal disease. In the absence of significant environmental variation this illustrates the power of genetic constitution in determining resistance or susceptibility in an outbred population. Attempts to establish a model of more acute and progressive disease should consider incorporating a protein-deficient diet that is more representative of the diets of dogs in endemic areas. Second, the overall mortality was relatively low (23.8%) and the dogs that survived showed initial evidence of disease that then regressed, either partially or completely. Third, the data confirm that transmission of *L. chagasi* from most infected dogs to the sand fly vector is an inefficient process in that only a small proportion of infected dogs that has progressive disease and high parasite burdens probably contribute to the bulk of parasite transmission. Fourth, surrogate markers of disease such as hematocrit and immune response were highly variable and offered little additional data over clinical evaluation. Fifth, the measurement of the parasite burden may offer a useful surrogate marker of disease, but more sensitive and quantitative measurements (e.g., real-time PCR) are likely to offer an advantage over the aspirate-culture method used in this study. Finally, the high degree of variability in disease evolution and among markers of disease in the outbred population will necessitate large sample sizes for future vaccine or other intervention studies.

<table>
<thead>
<tr>
<th>Vaccine efficacy endpoint</th>
<th>Current data</th>
<th>Targeted vaccine-induced reduction</th>
<th>Estimated sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease prevalence (proportion of polysymptomatic months over 1 year of observation)</td>
<td>26/132 31/120</td>
<td>50% 109</td>
<td>82</td>
</tr>
<tr>
<td>Disease prevalence (mean ± SD polysymptomatic months over 1 year of observation)</td>
<td>2.7 ± 3.3 2.9 ± 4.3</td>
<td>70% 37†</td>
<td></td>
</tr>
<tr>
<td>Mortality rate (1 year)</td>
<td>3/10</td>
<td>50% 194* 106*</td>
<td></td>
</tr>
<tr>
<td>Splenomegaly (proportion of animals with splenomegaly evident at necropsy)</td>
<td>3/8 1/5</td>
<td>70% 89* 51*</td>
<td></td>
</tr>
<tr>
<td>Parasite detection (proportion of animals with parasites detected in LN at 4 mo p.i.)</td>
<td>1/11 5/9</td>
<td>80% 136* 17*</td>
<td></td>
</tr>
<tr>
<td>Infectivity of dogs to sand flies (proportion of positive animals between 4–6 mo p.i.)</td>
<td>0/11 3/9</td>
<td>90% 100* 12*</td>
<td></td>
</tr>
</tbody>
</table>

Sample size in the table was calculated by the proportion of percent vaccine-induced reduction with the significant level of 5%, two-sided test, and 80% of power to detect the reduction (t2 test).

† Was calculated by one-sample T-test with the significant level of 5%, two-sided test, and 80% of power to detect the reduction.

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