Quantification of the infectious dose of *Leishmania major* transmitted to the skin by single sand flies

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Leishmaniasis is transmitted between mammalian hosts by the bites of bloodsucking vector sand flies. The dose of parasites transmitted to the mammalian host has never been directly determined. We developed a real-time PCR-based method to determine the number of *Leishmania major* parasites inoculated into the ears of living mice during feeding by individual infected flies (*Phlebotomus duboscqi*). The number of parasites transmitted varied over a wide range in the 58 ears in which *Leishmania* were detected and demonstrated a clear bimodal distribution. Most of the infected mice were inoculated with a low dose of <600 parasites. One in four received a higher dose of >1,000 and up to 100,000 cells. High-dose transmission was associated with a heavy midgut infection of >30,000 parasites, incomplete blood feeding, and transmission of a high percentage of the parasite load in the fly. To test the impact of inoculum size on infection outcome, we compared representative high- (5,000) and low- (100) dose intradermal needle infections in the ears of C57BL/6 mice. To mimic natural transmission, we used sand fly-derived metacyclic forms of *L. major* and preexposed the injection site to the bites of uninfected flies. Large lesions developed rapidly in the ears of mice receiving the high-dose inoculum. The low dose resulted in only minor pathology but a higher parasite titer in the chronic phase, and it established the host as an efficient long-term reservoir of infection back to vector sand flies.

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Leishmania parasites have a digenetic life cycle that alternates between primarily intracellular amastigotes residing in the blood and tissues of mammalian hosts, and extracellular stage promastigotes that inhabit the guts of vector sand flies (1). An infected sand fly becomes competent to transmit infection after growth, differentiation, and anterior migration of parasites in the midgut, accompanied by the extensive attachment of promastigotes to the walls of the thoracic midgut and to the stomodeal valve (2). The stomodeal valve is found at the anterior end of the midgut and forms part of the system of pumps used in blood sucking. The lumen is also packed with parasites embedded in a parasite-derived matrix, the promastigote secretory gel (PSG) (3). Many of these cells are the metacyclic forms that are preadapted to survival in a subsequent mammalian host and are therefore considered the infective form (4, 5).

Mice are routinely infected experimentally with *Leishmania* by s.c. or intradermal needle injections of 10^2 – 10^7 parasites. It is not clear how well the resultant disease mimics that caused by natural transmission from sand flies. In particular, some components of both sand fly saliva and PSG are believed to influence the course of disease keenly when coinjected into the wound during feeding (6, 7). Moreover, the number of *Leishmania* cells injected by an infected sand fly during natural transmission is not known. The best estimate to date was reported by Rogers *et al.* (6) at 10^3 per biting fly. In their study, the dose delivered by groups of *Lutzomyia longipalpis* sand flies infected with *Leishmania mexicana* (an unnatural but permissive combination) was

calculated as the average number of parasites collected in the culture medium per fly observed to feed at the membrane, an approach that was insufficient to determine the variation in the numbers delivered by individual flies. Questions also arise over potential alterations in the behavior of flies feeding on an artificial apparatus rather than a living host and on culture medium rather than blood.

In the present work, we have determined the number of *Leishmania major* parasites transmitted by individual infected sand flies allowed to feed on living mice. Our real-time PCR-based approach revealed a wide variation in the size of the inocula delivered. Needle infection studies designed to mimic transmission by sand flies showed clear differences in the progression of disease and the chronicity of infection initiated with a representative “high”- or “low”-dose inoculation. The variation in infectious dose delivered by vector sand flies is therefore likely to be an important, and largely overlooked, determinant of disease outcome.

Results

Groups of sand flies (*Phlebotomus duboscqi*) were infected with *L. major*, an agent of zoonotic cutaneous leishmaniasis. When the infections had matured (13–19 days), individual flies were allowed to feed on ears of anesthetized BALB/c mice. To quantify the number of *Leishmania* parasites transmitted, we used a *Leishmania* rDNA-specific real-time PCR. Because the quantity of mouse tissue used in the DNA preparation proved to be a limiting factor in the detection of far less abundant *Leishmania* DNA, adhesive tape was used to restrict the area of the ear available to the flies to a 5-mm diameter circle of skin. After 1–3 h of exposure of each ear to a single infected fly, the mice were killed, and the exposed region of the ear was excised and processed to extract template DNA for a real-time PCR. By comparison with a set of standards made using unbitten mouse tissue and known numbers of *Leishmania* cells grown in culture, we could consistently detect the DNA from as few as 10 *Leishmania* parasites coprocessed with mouse tissue [supporting information (SI) Fig. S1]. After their feeding attempt, we also dissected each of the infected flies and recorded the following parameters to compare with the transmission data: (i) the size of any blood meal taken, (ii) the distention of the crop with stored sugar solution, and (iii) the number of promastigotes remaining in the gut, including the percentage that were metacyclic forms.

The normal variation among coinfecting flies meant that some of those used in our experiments had light or immature infec-

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Table 1. Summary of fly infections, blood feeding, and transmission results

Group	Strain	Age of fly infection, days	No. of flies	No. with visible blood meal	Average prefeeding parasite load*	Low-dose transmitters†	High-dose transmitters‡
1	FV1	13	20	9	14,100 ± 10,200	3	2
1	FV1	15	20	5	35,800 ± 11,200	9	2
1	FV1	19	15	8	18,100 ± 20,700	0	1
2	FV1	14	25	6	17,600 ± 5,500	1	0
2	FV1	15	25	8	32,300 ± 11,500	4	2
3	Sd	14	25	10	55,900 ± 22,300	1	0
4	FV1	13	23	9	21,400 ± 7,400	4	1
4	FV1	15	20	3	23,900 ± 11,000	1	0
5	FV1	13	24	7	17,400 ± 6,300	3	0
5	FV1	15	12	3	27,000 ± 15,600	1	0
6	Sd	13	14	0	32,700 ± 10,700	0	0
6	Sd	16	30	5	25,500 ± 13,700	1	0
7	FV1-RFP	13	24	5	54,700 ± 17,600	10	3
7	FV1-RFP	14	24	2	77,300 ± 24,600	6	3
Total			301	80		44	14

*Midgut load was calculated by the addition of the midgut parasite count after feeding to the value of the detected inoculum.

† Fewer than 600 parasites.

‡ More than 1,000 parasites.

tions that were rarely transmitted. To improve the efficiency with which single fly transmission was achieved, we developed an immunofluorescence-based system to track the progress of *Leishmania* infections in living flies. We imaged whole sand flies infected with a line of *L. major* FV1 parasites transfected with a plasmid containing a red fluorescent protein gene (FV1-RFP). This brightly fluorescent cell line grows normally in culture and produces infections in sand flies similar to wild type (N.P., N.K., N.S., P.L., and D.S., unpublished observations). Flies were selected based on the position of the signal in the anterior midgut (Fig. S2A) and on the strength of the signal ($>2.5 \times 10^6$ fluorescence units), which, as shown in Fig. S2B, is directly correlated with midgut infection intensity.

Table 1 summarizes the results of 14 independent experiments using seven groups of infected flies. *Leishmania* parasites were detected in a total of 58 of 301 mouse ears, each exposed to a single infected fly. Many of the 301 flies took no visible blood meal and may not have attempted probing or feeding on the mouse, providing no opportunity for transmission. Surprisingly, when transmission occurred, the size of the inoculum varied widely from <10 to nearly 100,000 *Leishmania* cells (Fig. 1A). Interestingly, there was a clear bimodality to the inoculum size distribution. A Gaussian mixture cluster analysis on the log-transformed counts detects two clusters with normal distribution and equal variance: a high-dose cluster in which 14 of 58 infected mice received 1,000 or greater parasites (geometric mean, 8,775; 95% C.I., 3,939–19,547; median, 7,995), and a low-dose cluster in which 44 of 58 mice received 600 or fewer parasites (geometric mean, 42; 95% C.I., 30–60; median, 37).

The Gaussian mixture model cluster analysis was also used to include both transmitted dose and the prefeed parasite load. This two-dimensional analysis also detects two clusters: a high-prefeed and high-transmission cluster ($n = 14$) and a high-prefeed and low-transmission cluster ($n = 40$) (Fig. 1B). Note that there are four flies in which the prefeed load is not available. For the 14 flies that are in the high-transmission cluster, there is a strong correlation between prefeeding load and number of cells transmitted (Spearman's correlation, 0.754; $P = 0.0027$; 95% C.I., 0.435–1). By contrast, for the low-transmission cluster there is no significant correlation between prefeeding load and number of cells transmitted (Spearman's correlation, 0.123; $P = 0.448$; 95% C.I., -0.200 to 0.447). The analysis also reveals that prefeeding parasite load of $>30,000$ promastigotes appears to be

a precondition for transmission of a high-dose inoculum. This load is not sufficient in all cases, however, because 56% of the transmitting flies that had this intensity of mature, midgut infections delivered a low-dose inoculum. The high-dose-transmitting flies also harbored a higher proportion of metacyclic promastigotes (mean, 30.7%; 95% C.I., 20–41.4) compared with the low-dose transmitters (mean, 16.2%; 95% C.I., 11.4–21) (Fig. S3A). The percentage metacyclics was in general positively correlated with infection intensity (Fig. S3B). Note that the overall frequency of transmitting flies that delivered a high-dose inoculum (25%) may have been skewed slightly upward by the

Fig. 1. Summary of transmitted dose and midgut infection intensity of transmitting flies. (A) Pooled data showing the number of *L. major* parasites, determined by real-time PCR, that were transmitted to BALB/c mice by individual infected *P. duboscqi* sand flies allowed access to a section of mouse ear for up to 3 h. (B) Pooled data showing the relationship between the number of *L. major* parasites in the gut of *P. duboscqi* sand flies and the infectious dose transmitted by those flies. Two-dimensional Gaussian mixture cluster analysis detects two clusters, identified by different symbols, and plotted together with mean and associated ellipse, representing a two-dimensional analog of the standard deviation. There was a strong correlation between prefeeding parasite load and the dose of transmitted parasites among the 14 flies in the high prefeed, high-dose cluster (Spearman's correlation, 0.754; $P = 0.0027$; 95% C.I., 0.435–1).

Fig. 2. Transmitted dose as a function of midgut infection intensity. Pooled data showing the relationship between the prefeeding parasite load in the gut and the percentage of the load delivered. Squares, low-dose transmitters; triangles, high-dose transmitters.

two experiments involving the preselected FV1-RFP *L. major*-infected flies that had stronger prefeeding parasite loads relative to the average of the nonselected groups and for which the proportion of transmitting flies that delivered a high dose was 32%.

One of the most distinctive features of high- vs. low-dose-transmitting flies is not the parasite load in the fly *per se*, but rather the percentage of that load that was transmitted. The 14 high-dose-transmitting flies delivered a geometric mean of 14% (95% C.I., 7.4–25.4) of their prefeeding parasite load to the mouse. Low-dose-transmitting flies by contrast delivered only a geometric mean 0.15% (95% C.I., 0.09–0.25) of their total parasite load (Fig. 2). Interestingly, the linear trend in the high-dose transmitters was delivery of a greater proportion of their parasites as prefeeding loads increased, whereas in the low-dose transmitters an opposite trend was observed. The clearest outlier in these data was again the single poorly infected fly (prefeeding parasite load of 4,200) that transmitted 600 promastigotes, or 14% of its initial load.

In contrast to uninfected sand flies, *Leishmania*-infected flies often have difficulty taking blood meals and fail to fully engorge. In the present work we detected an inverse linear trend between prefeeding parasite load in the flies and the size of blood meal taken (Fig. 3A; Jonckheere–Terpstra test, $P = 0.0002$). Infected flies without visible blood meals were not included in this particular analysis because, with the exception of the flies that transmitted parasites, it is not known whether the others attempted to feed. The number of parasites transmitted by flies that took visible blood meals was also inversely associated with the size of the blood meal taken (Fig. 3B; Jonckheere–Terpstra test, $P = 0.019$). All but one of the 14 flies transmitting a high-dose inoculum demonstrated compromised feeding success, taking one-half or less of a full blood meal. Note that blood feeding was not a strong predictor of overall transmission success because more than half of the transmitting flies took no visible blood meal, and 71% of the flies with a visible blood meal did not transmit parasites.

Finally, there was no correlation between the amount of sugar stored in the crop immediately after feeding and either the total parasite load in the flies or their ability to transmit high or low doses of *L. major* (Fig. S4).

The wide variation in inoculum size suggests that the infectious dose delivered by vector sand flies may be a determining factor in the outcome of clinical disease. We wanted to compare lesion severity and parasitic load in mice infected by needle by

Fig. 3. Relationship among parasite load, blood meal engorgement, and the infectious dose transmitted to the mammalian host. (A) Pooled data showing the parasite load in sand flies that took different sizes of blood meals. Flies with greater parasite loads took smaller, partial meals: Jonckheere–Terpstra test, $P = 0.0002$. (B) Pooled data showing the infectious dose transmitted by sand flies that took different sizes of blood meals. Flies that took smaller blood meals tended to transmit a larger infectious dose: Jonckheere–Terpstra test, $P = 0.019$.

using doses approximating the median numbers of metacyclics inoculated by the high- and low-dose-transmitting flies (5,000 and 100, respectively). To mimic further the conditions that might influence the outcome of sand fly-transmitted infections, the metacyclic promastigotes used for challenge were purified from the sand fly midgut and were injected into the ear dermis immediately after exposure of the injection site to the bites of uninfected flies. The high-dose inoculum produced a rapid onset of lesions that peaked 3–4 weeks after challenge and that resolved gradually over the subsequent 10–12 weeks (Fig. 4A). By contrast, the low-dose inoculum produced consistently smaller lesions that were delayed in onset but that also required a long time to resolve fully (3–4 months). The rapid development of lesions in the high-dose group was associated with a log fold higher number of parasites in the inoculation site 9 days after infection (Fig. 4B). By day 45, the numbers were approximately equivalent, and by day 101 the low-dose group actually harbored

A repeat experiment was designed primarily to compare parasite persistence in the chronic phase with respect to their potential for transmissibility back to the vector. Mice that were challenged as above again demonstrated an early onset and rapid development of large lesions after infection with 5,000 sand fly-derived metacyclics, whereas 100 parasites again produced only minor pathology throughout the course of infection (data not shown). The sampling of healed lesions at day 151 with 9–10 flies per ear revealed, as shown in Fig. 4C, that the ears harboring persisting parasites after resolution of low-dose infections had a slightly greater, although nonsignificant, chance of transmitting parasites to a fly than the high-dose infections [16.3% transmitting for low dose vs. 11.1% transmitting for high dose, odds ratio (OR), 1.56; 95% C.I., 0.27–9.18; $P = 0.579$].

Discussion

The dose of *Leishmania* promastigotes delivered by the sand fly vector to the skin of the mammalian host has never been directly determined. The only previous studies to quantify the numbers of promastigotes egested by infected sand flies used artificial feeding systems (6, 8). Although providing the best estimates to date, these approaches failed to account for normal probing and feeding behavior or the number egested by a single fly as opposed to the average number determined from collective feeds. Using a real-time PCR approach optimized to detect as few as 10 *L. major* promastigotes deposited into the mouse ear dermis, we were able to reveal a remarkable range (10–100,000) in the dose of parasites transmitted to mice exposed to a single infected sand fly. The dose distribution was distinctly bimodal, with $\approx 75\%$ of the transmitting flies delivering 600 or fewer promastigotes, and the remainder delivering >1000 cells. This broad dose range is similar to that determined from single flies force-fed into microcapillaries (8), with the exception that a bimodal distribution was not reported for the fewer transmitting flies studied with this technique. Although we believe that this bimodality reflects fundamentally different mechanics of transmission, it is of course possible that sampling a larger series of transmitting flies would reveal a more continuous dose size distribution. Furthermore, although we have been careful to employ a *Leishmania*–sand fly–rodent host combination that is consistent with a natural transmission setting, we can only speculate that the remarkable range in the number of parasites delivered by individual infected flies occurs in the wild or with transmission involving different species of parasites, vectors, and reservoir hosts.

Among the transmitting flies, there was a clear set of parameters associated with their ability to deliver a high vs. low dose of parasites. Those predictive of high-dose transmission were prefeeding parasite loads of >30,000 promastigotes, an inability to engorge fully, and egestion of a relatively high proportion of the parasites in the gut. Furthermore, above the apparent threshold necessary for the transmission of a high-dose inoculum, there was a direct correlation with increased transmitted dose and the infection level in the midgut. Each of these conditions is consistent with the “blocked-fly” hypothesis, proposed by Shortt and Swaminath in 1928 (9) and refined in the context of parasite-secreted molecules that contribute to the formation of a biological plug (4). It has been consistently noted that heavily infected sand flies experience difficulty in feeding (10–12), which is associated with the stomodeal valve being forced open by the pressure of the plug (4) and/or by direct damage to the valve as a consequence of a chitinase secreted by attached promastigotes (13–15). From the current data, it seems clear that an especially heavy anterior midgut infection promotes the conditions under which a substantial fraction of the parasites can become mobilized for egestion, possibly associated with a reflux of parasites during attempts by the fly to dislodge the plug. Whatever the precise mechanism, these occasional high-dose

deliveries reinforce the conclusion from Bates and Rogers (16) that such high numbers cannot be accounted for by parasites originating in the foregut. Although it is possible that in some instances the high doses reflect additive deliveries associated with multiple feeding attempts, it seems unlikely that repeated deliveries of a typical low-dose inoculum would ever achieve most of the high-dose transmissions observed. It is important to emphasize, nonetheless, that the doses observed reflect transmission by single flies and not necessarily by single bites.

Low-dose deliveries accounted for the majority of successful transmissions and occurred in flies harboring high or low parasite loads in the midgut or that took full, partial, or no blood meals at all. It may be significant that the proportion of the prefeeding parasite loads delivered was, with only one exception, extremely low. This finding suggests that parasites in the foregut or associated with the anterior aspects of the open valve may have been more passively transmitted as a result of the backflow of the ingested meal rather than a more forceful regurgitation of parasites from behind the stomodeal valve. This may occur, as suggested by Schlein *et al.* (13), when the parasites disrupt the coordinated action of the pharyngeal and esophageal pumps or even when the feeding pumps are functioning normally. Thus, the source of low-dose inocula is expected to be flies that experience interrupted feeding and abandon their feeding attempts or that ultimately succeed in engorgement without dislodging parasites in the plug.

The revelation of such great variability in transmitted dose emphasizes the potential role of dose along with species/strain and host factors in determining the clinical outcome of *Leishmania* infection in the host. In particular, the findings suggest that severe pathologies might be the result of a very high-dose inoculum, whereas subclinical infections, which account for a high proportion of *Leishmania* exposures in humans (17–19), might be the result of infected flies delivering a number of parasites below the threshold required to produce overt histopathological changes in the host. We were unable to determine which mice received a high- or low-dose inoculum without killing the animals at the time of exposure to infected flies, so we attempted to mimic the conditions associated with sand fly delivery of high- and low-dose inocula by using purified, sand fly-derived metacyclic promastigotes, injected by needle into mouse ears immediately after exposure of the injection site to the bites of uninfected flies. Assuming, based on prior studies (6, 8, 20), that the majority of fly-transmitted parasites are metacyclic promastigotes, needle injection of 5,000 sand fly-derived metacyclics produced lesions that were rapid in onset and relatively severe in size and duration but that eventually healed. By contrast, 100 metacyclics produced only slight pathology, but interestingly, and consistent with a prior study (21), the number of parasites persisting in the skin during the chronic phase was higher in the mice receiving the low-dose challenge. We were able to demonstrate formally that the low-dose infection established the host as a long-term reservoir of infection back to the vector, at least as efficient as the mice receiving the high-dose challenge. Thus, the majority of *Leishmania* infections in their mammalian hosts may be the result of low-dose exposures that lead to no or minimal disease, yet establish these hosts as a long-term reservoirs of infection to vector sand flies. The wide dose range transmitted by single flies poses an important question as to the physiologic dose chosen for experimental studies and, in particular, for evaluation of vaccine efficacy. Because the primary end point for any clinical vaccination study against *L. major* will be cutaneous pathology, then the typical high dose of 5,000–10,000 metacyclic promastigotes would seem to be an appropriate inoculum size. It is important to note that this dose is still 50- to 2,000-fold lower than the needle inocula used in the challenges for the vast majority of the reported *L. major* vaccination studies in mice. It is also important to emphasize that even

an appropriate dose or range of doses injected by needle will not take into account the virulence factors that have been suggested to accompany sand fly-transmitted infections (6, 7). Ultimately, infected sand flies provide the most meaningful test of vaccine efficacy, but they have only been used as such in one instance (22), so far as we are aware. Their use has been constrained by the availability of laboratory-reared flies and the difficulty in getting infected flies to transmit infection by bite reliably. As we have shown, RFP-expressing *L. major* parasites can be used as a means of preselecting the infected sand flies most capable of transmission by bite, such that low numbers of infected flies can be used in *Leishmania* challenge studies to reproduce the most basic elements of natural exposure efficiently.

Materials and Methods

***Leishmania* Cell Lines.** Transmission experiments were carried out by using three lines of *Leishmania*; the *L. major* Friedlin strain from the Jordan Valley, National Institutes of Health (NIH)/FV1 (MHOM/IL/80/FN); the West African *L. major* Seidman strain, NIH SD (MHOM/SN/74/SD); and *L. major* FV1-RFP, the FV1 strain transfected with a plasmid containing a RFP gene, generated as follows. The DsRed gene was amplified by PCR using the pCMV-DsRed-Express plasmid (BD Biosciences/Clontech) as template and the forward primer 5'-TGG ACT AGT ATG GCC TCC TCC GAG GAC GTC-3' and reverse primer 5'-CCA ACT AGT CTA CAG GAA CAG GTG GTG GCG-3'. The PCR product was first cloned into the pCR2.1 plasmid (Invitrogen) and the sequence verified by nucleotide sequencing. The Spel insert from a selected clone was subsequently ligated into the Spel site of the pKSNEO *Leishmania* expression plasmid (23). FV1 promastigotes were transfected with the resulting expression plasmid construct pKSNEO-DsRed by electroporation and selected for growth in the presence of 50 μ g/ml Geneticin (G418) (Sigma), as described in ref. 24.

Mice. BALB/c and C57BL/6 mice were purchased from the Division of Cancer Treatment, National Cancer Institute, NIH. All mice were maintained in the National Institute of Allergy and Infectious Diseases animal care facility under specific pathogen-free conditions.

Exposure of Mouse Ears to Infected Sand Flies. Two- to 4-day-old *P. dubosqi* females were obtained from a colony initiated from field specimens collected in Mali. They were infected by artificial feeding through a chick skin membrane on heparinized mouse blood containing 4×10^5 *L. major* amastigotes per ml, excised, and purified from BALB/c footpad lesions and stored frozen until use. After 13–19 days, individual flies were transferred to small plastic vials (3-dram volume, 4.8-cm height, 1.8-cm diameter) covered at one end with a 0.25-mm nylon mesh. BALB/c mice were anesthetized by i.p. injection of 30 μ l of ketamine/rompin (100 mg/ml). The inside of each ear of the mice was covered with a small piece of adhesive tape with a 5-mm diameter hole stamped out from the middle. Specially designed clamps were used to hold the mesh end of each vial flat against the ear of a mouse so that the fly had access to feed on the exposed 5-mm-diameter circle of skin for a period of 1–3 h in the dark. The mice were then killed, and a 6-mm biopsy punch was used to excise the exposed region of ear. The corresponding flies were dissected, recording the size of any blood meal in the gut and sugarmal in the crop. Each gut was transferred to 30 μ l of PBS in a microfuge tube, macerated with a plastic pestle (Kimble Chase), and the number of metacyclic and nonmetacyclic forms (distinguished by morphology and movement) was determined by counting on a hemocytometer.

Fluorescence Microscopy for Preselection of Infected Flies. *L. major* FV1-RFP-infected flies were anesthetized with carbon dioxide gas and transferred to individual wells of a flat-bottomed, black-walled 96-well plate that was precooled at -20°C and kept on an ice pack to ensure the flies did not awake during microscopy. Each fly was scored for the intensity of fluorescence emitted from the anterior midgut when viewed under an Olympus OV-100 fluorescence microscope. Only flies emitting strong fluorescence in the anterior midgut ($>2.5 \times 10^6$ fluorescence units), quantified by using ImageJ 1.37v (Wayne Rashband, NIH, <http://rsb.info.nih.gov/ij/>) were used in subsequent transmission experiments.

DNA Preparation. Six-micrometer-diameter punches of mouse ear tissue that had been exposed to infected flies were placed in 500 μ l of lysis buffer [200 mM NaCl, 100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 500 μ g/ml proteinase K]. For each experiment, a new set of standard DNA samples was prepared alongside the test samples. They were made by using ear punches

from control, unexposed mice that were put in lysis buffer along with a known number of the corresponding strain of *L. major* parasites (10^1 – 10^5) obtained from culture. The samples were incubated at 56°C for 12–16 h with occasional agitation to break up the tissue. The proteinase K was inactivated by 10 min at 95°C. After cooling slightly, ice-cold potassium acetate was added to 1 M from a freshly prepared 10× stock, and the samples were kept on ice for 1 h to precipitate the SDS. Samples were spun at 16,000 × *g* for 10 min, and the supernatant was carefully removed to a new tube. The DNA was precipitated with equal volumes of isopropyl alcohol, pelleted by a 10-min spin at 16,000 × *g*, washed in 70% ethanol, dried briefly, and then resuspended in 100 μl of water. The DNA concentration of each sample was measured with a Nanodrop ND-1000 spectrophotometer.

Real-Time PCR. Real-time PCR on each DNA sample was run twice in triplicate on an ABI Prism 7900HT sequence detection system (Applied Biosystems). Each 20-μl reaction contained 10 μl of Universal PCR Mastermix (Applied Biosystems), 3 mM MgCl₂, 0.1 mg/ml BSA, 10 μM primer U1 (AAG TGC TTT CCC ATC GCA ACT), 10 μM primer L1 (GAC GCA CTA AAC CCC TCC AA), 0.4 μM probe Leish P1 (6FAM-CGG TTC GGT GTG TGG CGC C-TAMRA), 10 ng of human DNA, 0.75 μl of RNase P control reagents (VIC) (Applied Biosystems), and 1 μl of the sample template DNA. Each reaction was multiplex, with amplification from the RNase P primers and human template DNA acting as a control for reaction efficiency. A sample was recorded as positive for the detection of *Leishmania* DNA if the fluorescence emitted by the Leish P1 probe, which recognizes *Leishmania* rDNA, exceeded the automatic threshold within a cutoff 40 cycles in five of the total six-reaction run. Quantification was by automatic comparison with the specific set of standard samples prepared in parallel to each set of test samples. The number of *Leishmania* cells in each sample was determined as the mean of the two median values from the three reactions in each run.

High- and Low-Dose Needle Infections. To mimic natural transmission from sand flies, C57BL/6 mice were injected by needle with metacyclic promastigotes isolated from *P. dubosqi* sand flies infected for 13–15 days with *L. major* clone FV1. Dissected guts were macerated briefly with a plastic pestle and then spun twice at 80 × *g* for 1 min to remove the debris. The cells in the supernatant were washed in 2 ml of DMEM, resuspended in 0.5 ml of DMEM containing 100 μg/ml peanut agglutinin (Vector Laboratories), incubated for 5 min, then spun for 8 min at 80 × *g* to remove the agglutinated, nonmeta-

cyclic forms. The metacyclic cells in the supernatant were washed, counted, and volumes adjusted for 5.0-μl injection of 5,000 or 100 parasites into the mouse ears. The ears of the mice were exposed for the 2 h immediately preceding injection to five uninfected flies per vial/ear as described above, including the use of adhesive tape to restrict the region of ear exposed to the flies. The fly-derived parasites were injected into the center of the exposed circle of skin, thus placing the infection in close proximity to the uninfected fly bites. Infections were monitored by weekly measurement of the longest diameter of any lesions that appeared on the ears by using calipers. At intervals, some of the mice were killed to estimate the parasite load at the site of infection, as described in ref. 25, by serial dilution of the ear tissue homogenate in a NNN growth medium containing 20% defibrinated rabbit blood.

Transmissibility of Parasites in Chronic Lesions Back to Vector Sand Flies. Ears of C57BL/6 mice harboring persistent infections with *L. major* FV1 were exposed to 10 uninfected flies per vial/ear as described above, with the exception that the entire ear was exposed to the flies. Blood-fed flies were separated at 24 h after feeding, and their midguts were dissected at 72 h and examined microscopically for the presence or absence of promastigotes.

Statistical Methods. We used Gaussian mixture model-based cluster analyses on the number of transmitted cells either alone or together with the prefeeding load. These analyses assume that the log-transformed responses are a mixture of normal (i.e., Gaussian) distributions, in which neither the number of distributions nor the shape, volume, or orientation of each of those distributions is prespecified. The best-fit model is chosen by Bayesian information criteria without using a prior distribution (26). Student's unpaired *t* test was used to determine the statistical significance of differences between infection outcomes in mice challenged with high- and low-dose inocula. Differences in the probability of an individual fly picking up parasites from the infected mice was tested by using a logistic model with a sandwich estimator of variance to account for possible correlation within each mouse (27) adjusted for small samples. We used R version 2.6.1 (R Development Core Team, 2007, www.R-project.org) to do the logistic model, and R with the Mclust package (27) to do the cluster analysis. For the Spearman's correlation and Jonckheere–Terpstra tests we used Stat Xact 8 Procs (Cytel).

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