A Mouse Model of *Leishmania braziliensis braziliensis* Infection Produced by Coinjection with Sand Fly Saliva

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Summary

To development a reliable murine model of *Leishmania braziliensis braziliensis* infection, parasites were injected into BALB/c mice in the presence of phlebotomine sand fly salivary gland lysates, which have previously been shown to greatly increase the infectivity of *L. major* in mice. When injected with salivary gland lysates, *L. braziliensis braziliensis* produced progressively enlarging cutaneous nodules, containing many macrophages filled with *Leishmania* amastigotes. In contrast, *L. braziliensis* injected without gland extracts produced small and rapidly regressing lesions. Isoenzyme analysis, monoclonal antibodies, and the polymerase chain reaction with *L. braziliensis*-specific oligonucleotide primers and probes confirmed that parasites causing the lesions were *L. braziliensis*.

Leishmania braziliensis is an intracellular protozoan parasite transmitted by phlebotomine sand flies to persons living in Central and South America. *L. braziliensis* is divided into four subspecies: *L. braziliensis braziliensis*, *L. braziliensis guyanensis*, *L. braziliensis panamensis*, and *L. braziliensis peruviana*. The parasites usually cause a self-healing ulcer at the site of the bite. *L. braziliensis braziliensis* is of particular concern because mucocutaneous disease, with disfiguring lesions of the mouth and nose, may develop (1).

L. donovani, the cause of visceral leishmaniasis, and *L. mexicana* and *L. major*, which cause cutaneous leishmaniasis, all produce progressive lesions in susceptible BALB/c mice. These lesions are characterized by the presence of numerous macrophages filled with *Leishmania* amastigotes (2). Therefore, mice have been used extensively to study the immune response to and the effect of drugs on these species of *Leishmania*.

In contrast, *L. braziliensis braziliensis* has not produced lesions in BALB/c mice (3). *L. braziliensis* from Panama, presumably *L. braziliensis panamensis*, and *L. braziliensis peruviana* caused cutaneous lesions of BALB/c mice that were variable (4) and progressive (5), respectively. Because of the difficulties with the mouse model, *L. braziliensis* has most frequently been studied in the Golden hamster (3, 6), which has an immune system much less well characterized than that of the mouse and for which few immunological reagents are available.

Sand flies salivate into the skin while probing for a blood-meal and transmitting *Leishmania*. Sand fly saliva contains several substances with potent pharmacological activities including a potent vasodilator (7). The saliva of sand flies also enhances transmission of *Leishmania* parasites, as *L. major* parasites injected with sand fly salivary gland lysates formed lesions in mice that were much larger than those of control parasites injected without saliva (8). The lesions caused by *L. major* and saliva contained as many as 5,000-fold more parasites than controls, and in the presence of salivary gland material as few as ten *L. major* parasites caused a lesion (8).

Here we report that *L. braziliensis braziliensis* parasites caused progressive cutaneous lesions when injected with sand fly glands into BALB/c mice, while control parasites injected alone produced small and rapidly regressing cutaneous nodules.

Materials and Methods

Parasites. *Leishmania* were cultured in Schneider's drosophila medium or on blood agar plates. *L. braziliensis braziliensis* strain WR604 was isolated from a single cutaneous lesion of a girl living in the Corte de Pebro region of Brazil. The parasite was typed by isoenzymes as an *L. braziliensis braziliensis* by Dr. Richard Kreutzer (Youngstown State, Youngstown, OH). *L. braziliensis guyanensis* strain 151M, which was used for sequencing conserved regions of the kinetoplast minicircle DNAs (kDNAs), was isolated from a Brazilian man and typed by isoenzymes by Dr. William O. Rogers (Harvard University, Boston, MA). Reference strains used as con-

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trols for isoenzyme typing and for the PCR included M2903 for *L. braziliensis braziliensis* and PH8 for *L. mexicana amazonensis*.

Infection of Mice and Harvest. Groups of four to five BALB/c mice were injected subcutaneously in the hind footpad with either 10^6 *L. braziliensis braziliensis* (WR 604 strain) or 10^6 *L. braziliensis* mixed with the lysate of one half of one salivary gland of the sand fly *Lutzomyia longipalpis*. Lesion development was followed by measuring the thickness of the injected footpad with a vernier caliper compared with the thickness of the contralateral uninfected footpad (8). After 2–4 mo mice were killed and the number of parasites within the lesions was determined by limiting dilution analysis (8). Cultured parasites were typed by isoenzymes, monoclonal antibodies, and the PCR. For histology, the infected footpads and draining LN were removed, fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin or with Giemsa.

DNA Probe Analysis. To obtain primers for the PCR that were specific for *L. braziliensis* minicircle DNA and did not prime *L. mexicana* minicircles, kDNA was isolated from *L. braziliensis guyanensis* strain 151M, cut with MspI and cloned into the phagemid vector Bluescript. The conserved regions of three *L. braziliensis* kDNAs were sequenced and compared with the sequences of the conserved regions of five *L. mexicana* kDNAs (9). These *L. braziliensis* kDNA sequences will appear in the EMBL, Genbank, and DDBJ Nucleotide data base with the following accession numbers: X54470, X54471, and X54472. In Fig. 1, conserved regions of single *L. braziliensis* and *L. mexicana* minicircles are aligned and the location of *L. braziliensis*–specific primers and probe and *L. mexicana*–specific primers and probe indicated. *L. braziliensis* primers were LB1 = CAAGCCTCTTAGAGGCCT and LB2 = AAATCAAAAATGGCAT, and the *L. braziliensis*–specific probe was LB3 = TAATTGTGCACGGGGAGG. The *L. braziliensis* primers, although derived from *L. braziliensis guyanensis*, amplify DNA from other *L. braziliensis* subspecies but not from *L. mexicana*. The *L. mexicana*–specific primers were LM1 = CAGTTTCCACCGCCCGAGCCG and LM2 = AATAAATGGGTCCCGGCC, and the *L. mexicana* probe was pMAT13A = GTGGGGGAGGGGCGTTCT. Although pMAT13A has previously been used as a general *Leishmania* primer (10), this DNA segment is used here as an *L. mexicana* probe because it falls inside the *L. mexicana*–specific primers but outside the *L. braziliensis*–specific primers (Fig. 1).

For PCR, cultured *Leishmania* were pelleted and DNA extracted in 100 μl of 10 mM Tris, 10 mM EDTA, 1% Triton-X 100, and 100 μ/ml proteinase K for 2 h at 50°C (11). After heat inactivation, *Leishmania* target DNAs were amplified using the PCR, Taq polymerase, and the *L. braziliensis*–specific primers LB1 and LB2 or *L. mexicana*–specific primers LM1 and LM2 (Fig. 1). 30 cycles were performed of denaturation at 94°C for 30 s, annealing at 45°C for 120 s, and elongation 72°C for 12 s. PCR products were identified by dot blotting to Biotrans membranes, and hybridization with ^{32}P-kinased oligonucleotide probes using a 50°C temperature for annealing and washing with 2× SSPE and 0.5% SDS (SSPE is composed of 0.36 M NaCl, 20 mM NaH_2PO_4, 2 mM EDTA, pH 7.4 [reference 11]). Alternatively, dot bots of PCR products were hybridized with radiolabeled kDNAs of *L. braziliensis* and *L. mexicana*, generously donated by Dr. Mark Rodgers (Harvard University (10).

Isoenzyme Analysis. Cultured parasites were lysed and isoenzyme profiles prepared as described by Kreuster and Christensen (12). Reference strains run in parallel were *L. braziliensis* and *L. mexicana*. Malic enzyme (1.1.1.40) was chosen to discriminate New vs. Old World *Leishmanias*. 6-Phosphogluconic dehydrogenase (E.C. 1.1.1.44) was chosen to distinguish *L. mexicana* from *L. braziliensis* parasites.

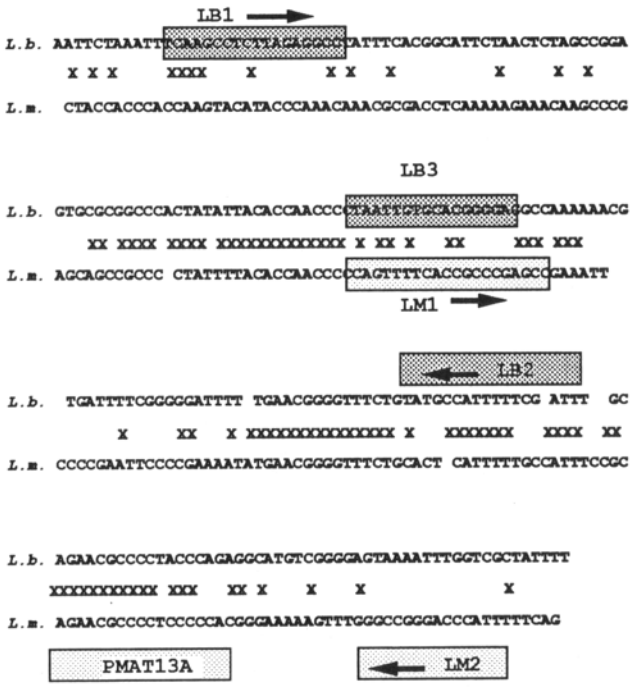

Figure 1. DNA sequence of the conserved region of the *L. braziliensis* (*L.b*) kinetoplast minicircle aligned with that of *L. mexicana* (*L.m.*). Xs mark identical bases between the two sequences. Blocks and arrows show location and direction of primers and probes specific for *L. braziliensis* or *L. mexicana* kDNAs. The sequences of the kDNA primers and probes are listed in Materials and Methods.

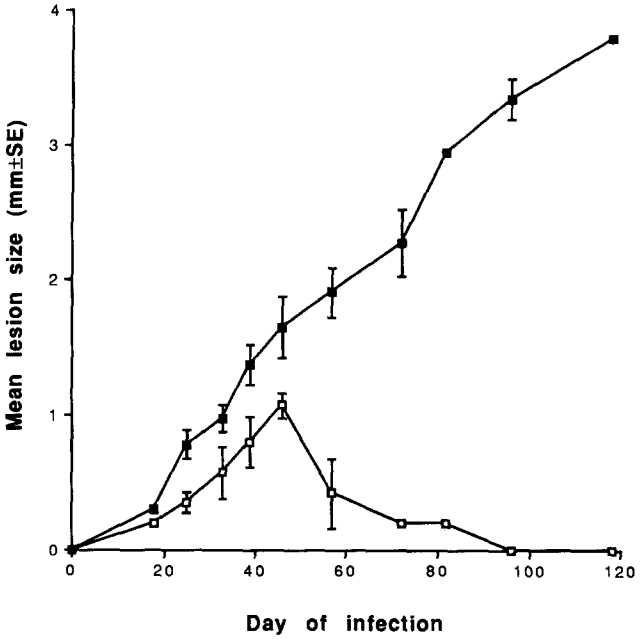

Figure 2. Enhancement of *L. braziliensis braziliensis* infection in BALB/c mouse footpads by co-injection of parasites with salivary glands lysates of *L. longipalpis*. Mice injected with one million *L. braziliensis braziliensis* parasites with the lysate of one half of a salivary gland (■) had lesions which grew larger with time and showed no evidence of resolution. In contrast, mice injected without salivary gland lysates (□) had lesions that were smaller and resolved with time.

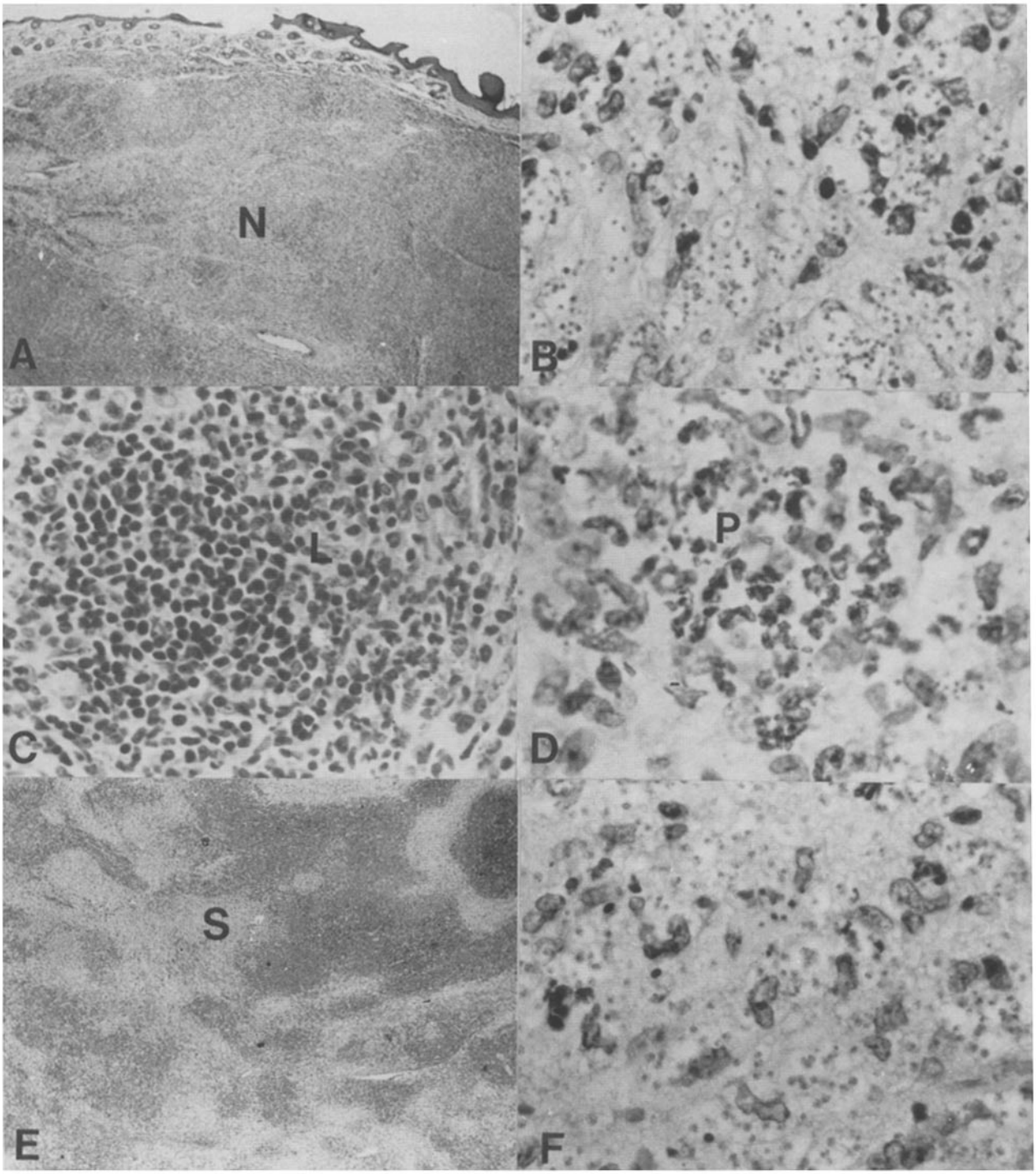

Figure 3. Histopathology of cutaneous mouse footpad lesions caused by *L. braziliensis braziliensis* parasites coinjected with sand fly salivary gland lysates. (*A*) At the site of the injection, there was a nodular infiltrate (*N*) that involved the dermis and the subcutaneous tissue. This nodule was primarily composed of sheets of macrophages within which were large vacuoles containing *L. braziliensis* amastigotes (*B*). There were also (*C*) focal accumulations of lymphocytes (*L*) and (*D*) microabscesses filled with polymorphonuclear leukocytes (*P*). (*E*) There was a marked sinus histiocytosis in the ipsilateral inguinal LN (*S*). (*F*) Within the expanded sinuses were macrophages containing *L. braziliensis* amastigotes. (*A* and *E*) × 100. (*B*, *D*, and *F*) ×1,800. (*C*) ×750.

mAb Analysis. Cultured parasites were dried onto glass slides, incubated with 1:100 dilutions of mAbs specific for *L. braziliensis braziliensis* (B-16), *L. braziliensis panmensis* (B-11), *L. braziliensis guyanensis* (B-19), *L. donovani* (D-2), and *L. mexicana* (M-9), generously donated by Dr. Diane McMahon-Pratt (Yale University, New Haven, CT) (13). Bound antibodies were detected with FITC-conjugated sheep anti–mouse antibodies and read blind with an Orthoplan fluorescence microscope (E. Leitz, Inc., Rockleigh, NJ).

Results and Discussion

Sand Fly Salivary Glands Dramatically Increase the Size of L. braziliensis braziliensis Cutaneous Lesions in Mice and the Number of Parasites Present. To develop a mouse model of *L. braziliensis braziliensis*, *Leishmania* parasites were injected into BALB/c mice in association with a salivary gland lysate of *L. longipalpis*, a sand fly vector for visceral leishmaniasis in South America. Coinjection of the sand fly saliva resembles natural *Leishmania* infection by means of a sand fly bite and has been shown previously to greatly increase the infectivity of *L. major* in mice (8). When *L. braziliensis* were injected into BALB/c mice with saliva, cutaneous lesions appeared after 3 wk and progressed with time (Fig. 2). In contrast, when *L. braziliensis* were injected without saliva, transient lesions formed that rapidly regressed (Fig. 2). Similarly, the number of parasites recovered from the BALB/c lesions caused by *L. braziliensis braziliensis* plus salivary glands after 8 wk was 20 ± 7 million (average ± SD), while <100 were recovered from *L. braziliensis* without saliva. Recently, we have also found that *L. longipalpis* salivary gland lysates enhance infection of BALB/c mice with *L. braziliensis braziliensis* strain Ltb111 and with *L. mexicana* strain PH8. Salivary gland lysates also produce larger lesions of *L. mexicana* in C3h mice, which are relatively resistant to this parasite.

Histopathy of L. braziliensis braziliensis Lesions in Mice Injected with Sand Fly Glands. *L. braziliensis braziliensis* injected with salivary gland homogenates produced cutaneous nodules, which involved the dermis and the underlying soft tissue of the BALB/c mouse footpad (Fig. 3 *A*). These nodules were filled with macrophages, each of which contained multiple *L. braziliensis braziliensis* amastigotes (Fig. 3 *B*). In addition there were focal accumulations of lymphocytes (Fig. 3 *C*) and occasional microabscesses composed of polymorphonuclear cells (Fig. 3 *D*). The draining inguinal LN were greatly enlarged and showed a marked sinus histiocytosis (Fig. 3 *E*). Within the sinuses were many macrophages containing *L. braziliensis braziliensis* parasites (Fig. 3, *F*). The footpad nodules in mice injected with *L. braziliensis braziliensis* and salivary glands are reminiscent of those caused by *L. mexicana* in susceptible mice (2). They are distinct from the lesions caused by *L. braziliensis braziliensis* in hamsters (6), which contain few parasites.

DNA Probe Analyses Show that the Parasites Recovered from the Mouse Lesions Are L. Braziliensis. To confirm that the *Leishmania* causing the progressive lesions in mice coinjected with sand fly salivary glands were caused by *L. braziliensis* and not by a stock of *L. braziliensis* contaminated with other species of *Leishmania*, parasites were isolated from mouse lesions and typed by DNA probes. Oligonucleotide primers and probes specific for *L. braziliensis* and of *L. mexicana* parasites were identified by comparing 300-bp-long conserved portions of the kinetoplast minicircle DNAs, which are highly repeated in the parasites and have previously been used to speciate *Leishmania* (Fig. 1 and reference 10). *Leishmania* from mice injected with or without salivary glands were confirmed to be *L. braziliensis*, because they gave a PCR product with the *L. braziliensis*–specific primers that was recognized by the radiolabeled *L. braziliensis*–specific oligonucleotide probe or by radiolabeled *L. braziliensis* kDNA (Fig. 4, *A* and *B*). Like the reference *L. braziliensis* strain M2903, the *L. braziliensis* recovered from the mouse did not produce a PCR product with the *L. mexicana*–specific primers recognized by pMAT13A oligonucleotide probe or by *L. mexicana* kDNA (Fig. 4, *C* and *D*). In contrast, *L. mexicana* reference strain PH8 DNA produced a PCR product recognized by the oligonucleotide

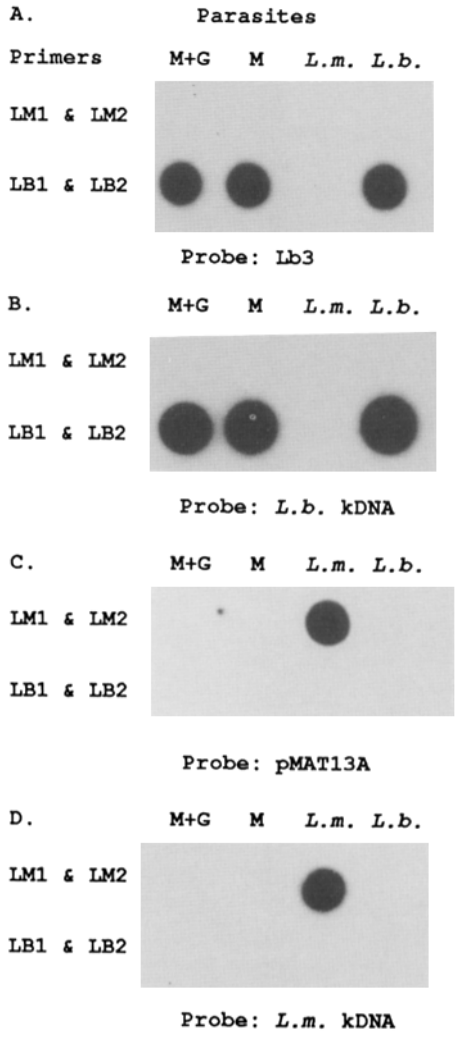

Figure 4. Dot blots of PCR products of *Leishmania* parasites with primers and radiolabeled probes specific for *L. braziliensis* or for *L. mexicana* kDNAs. Target DNAs were extracted from parasites recovered from mice injected with *Leishmania* strain WR604 plus glands (*M* + G) or alone (*M*), reference *L. mexicana* strain PH8 (*L.m*), and reference *L. braziliensis* strain M2903 (*L.b*). PCR were performed with *L. braziliensis*–specific primers LB1 and LB2 or with *L. mexicana*–specific primers LM1 and LM2 (Fig. 1 and Materials and Methods). PCR were probed with an *L. braziliensis*–specific oligonucleotide probe LB3 (*A*) or with *L. braziliensis* kDNA (*B*). Alternatively, PCR were probed with oligonucleotide pMAT13A, specific for *L. mexicana*, the PCR product of primers LM1 and LM2 (*C*), or with *L. mexicana* kDNA (*D*). This figure shows that WR604 parasites causing lesions in BALB/c mice were *L. braziliensis* and were not contaminated with *L. mexicana* parasites. This figure also shows that oligonucleotide probes rather than radiolabeled kDNAs can be used to detect the *L. braziliensis* or *L. mexicana* kDNA PCR products.

probe pMAT13A or by *L. mexicana* kDNA when PCRs were performed with *L. mexicana*–specific primers (Fig. 4, *C* and *D*). The *L. braziliensis*–specific primers did not produce a PCR product with the *L. mexicana* cells as a target (Fig. 4, *A–D*).

Because the PCR is able to greatly amplify small amounts of DNA, it is highly unlikely that any parasites other than *L. braziliensis* were present in the mouse lesions. There are two possible advantages of the PCR methods presented here for identifying *Leishmania* over previously published methods (10). First, the primers are specific for each species of *Leishmania* rather than priming all *Leishmania*. Second, oligonucleotides, which can be easily synthesized, are used as probes rather than kDNA, which must be purified from cultured parasites.

Isoenzymes and mAbs Also Confirm that the Leishmania Recovered from Mice Are L. braziliensis braziliensis. *Leishmania* before injection and after recovery from the footpads were typed by isoenzymes. *Leishmania* from the mice showed an identical migration of the malic enzyme as the *L. braziliensis braziliensis* and *L. mexicana* standards, which could be distinguished from the migration of malic enzyme from *L. major*. Further, the migration of 6-phosphogluconic dehydrogenase of the *Leishmania* recovered from the mice matched that of the *L. braziliensis braziliensis* standard and was distinct from that of the *L. mexicana* standard. These results confirm the DNA probe identification of the *Leishmania* that causes mouse lesions as *L. braziliensis*.

Leishmania recovered from the mice and the control *L. braziliensis braziliensis* strain M2903 both reacted positively with the mAb specific for *L. braziliensis braziliensis*, but did not react with mAbs specific for *L. braziliensis guianensis*, *L. braziliensis panamanensis*, *L. donovani*, or *L. mexicana*. Control *L. mexicana* organisms did not bind the *L. braziliensis braziliensis*–specific mAb. These results confirm the DNA probe and isoenzyme identification of the *L. braziliensis braziliensis* causing the mouse lesions.

Final Comments. The results presented here show that coinjection with sand fly glands dramatically increases the size and longevity of cutaneous *L. braziliensis braziliensis* lesions in BALB/c mice. To our knowledge, this is the first mouse model of *L. braziliensis braziliensis* infection. The mechanism by which sand fly saliva produces its effect is not known. Recent experiments suggest that the sand fly gland material may hinder the development of the mouse immune response to the parasites (14). Now that a mouse model showing progressive *L. braziliensis braziliensis* cutaneous lesions is available, these immunological questions can begin to be addressed. We are also investigating whether parasites isolated from human mucocutaneous lesions will produce mucocutaneous lesions in mice injected with the parasites plus salivary glands. If this is the case, a murine model for mucocutaneous disease will also be available.

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