A LABORATORY MODEL OF CANINE LEISHMANIASIS : the inoculation of dogs with *Leishmania infantum* promastigotes from midguts of experimentally infected phlebotomine sandflies

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Summary :

Twenty-five dogs (beagles) were infected with *Leishmania infantum* by the intradermal inoculation of an estimated 5-8,000 metacyclic promastigotes harvested from the midguts of 320 experimentally infected *P. perniciosus*. Details are given of the methods of infecting the flies and harvesting the parasites. All dogs developed small, self-healing chancres at the sites of inoculation. Parasites were isolated from lymph nodes, bone marrow or spleen of 21 dogs, 12 of which developed signs of disease and raised IFAT titres to *Leishmania*. Nine of the 21 remained healthy over a five-year observation period. Six of the nine were shown to have a cell mediated immune response to *Leishmania*. No parasites were isolated from four of the 25 dogs, two of which had a demonstrable cell mediated immunity and another had low transitory IFAT titres. The fourth had chancres at the sites of inoculation. The results show that dogs can be readily infected with promastigotes from the midguts of sandflies. However, a high proportion develop a cell mediated immunity and show no signs of disease. It is suggested that serological surveys of dogs for canine leishmaniasis reveal neither the true prevalence of infection nor the intensity of transmission. The efficacy of controlling human visceral leishmaniasis caused by *L. infantum* by destroying seropositive dogs is questioned.

KEY WORDS : canine leishmaniasis, dog, laboratory model, cell mediated immunity, humoral antibodies, *Leishmania infantum*, *Phlebotomus perniciosus*.

MOTS CLES : *leishmaniose canine, chien, modèle expérimental, immunité cellulaire, anticorps humoraux*, Leishmania infantum, Phlebotomus perniciosus.

Résumé : UN NOUVEAU MODÈLE DE LABORATOIRE : L'INOCULATION AU CHIEN DE PROMASTIGOTES DE *LEISHMANIA INFANTUM* OBTENUS DANS L'ESTOMAC DE PHLÉBOTOMES EXPÉRIMENTALEMENT INFECTÉS

Des doses de 5-8 000 promastigotes métacycliques de Leishmania infantum, *obtenus dans l'estomac de 320* Phlebotomus perniciosus *expérimentalement infectés, ont été inoculés en intra-dermique à 25 chiens de race Beagle. Les méthodes utilisées pour infecter les Phlébotomes et pour obtenir les parasites sont exposées en détail. Tous les chiens ont présenté un petit chancre spontanément guérissable au point d'inoculation. Les parasites ont été isolés à partir des ganglions lymphatiques, de la moelle osseuse ou de la rate de 21 chiens. Parmi ceux-ci, 12 ont développé des lésions apparentes et atteint en immunofluorescence des titres d'anticorps (IFAT) élevés et neuf sont restés en bon état durant les cinq années d'observation, mais six de ces neuf chiens ont eu une réponse immune à médiation cellulaire. Aucun parasite n'a pu être isolé de quatre des 25 chiens, deux d'entre eux ayant cependant une réponse immune à médiation cellulaire et un troisième un titre IFAT faible et transitoire.*

Ces résultats montrent que les chiens peuvent être aisément infectés avec les promastigotes d'estomacs de Phlébotomes. Cependant beaucoup développent une réponse immune à médiation cellulaire et ne montrent pas de signes cliniques. Cela suggère que les enquêtes sérologiques sur les chiens pour la leishmaniose canine ne révèlent ni la véritable prévalence de l'infection ni l'intensité de la transmission. La prophylaxie de la Leishmaniose viscérale humaine à Leishmania infantum *par destruction des chiens séropositifs est remise en question.*

There are two main difficulties in testing vaccines against canine leishmaniasis experimentally in dogs. The first is that dogs appear generally to respond to infection in one of two ways because of inherent differences in susceptibility (Lanotte *et al.*, 1979; Vidor *et al.*, 1991; Dye *et al.*, 1992; Cabral *et al.*, 1992; Pinelli *et al.*, 1994) which are evident by the immune response. A cell mediated immunity is associated with inapparent infections, whereas there is no evidence of protection by circulating antibodies (Liew and O'Donnell, 1993) constantly high levels of which usually indicate susceptibility with, eventually, signs of disease invariably followed by death if no treatment is given. Observations by Lanotte *et al.* (1979) and Pozio *et al.* (1981) on naturally infected dogs suggest that a few do not fall exactly into these categories. Some initially have high antibody titres which then fall, presumably as a cell mediated immune response develops, and such dogs may never show clinical signs of disease; others at first seem to be resistant, probably because of a cell mediated immune response that, however, may occasionally break down and be followed by rising antibody titres and clinical signs. In vaccine trials, known susceptible dogs should be used but, at the moment, there is no way to select them.

The second problem is to give a standard, repeatable challenge that will result in a predictable infection in unvaccinated, control dogs. Infecting dogs by the bite of experimentally infected sandflies is not only technically difficult, but also has the disadvantage that it

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is neither standard nor repeatable. It is unlikely that infected flies deposit a constant number of metacyclic promastigotes at each infecting bite. Promastigotes from the stationary phase of cultures of *Leishmania infantum* Nicolle appear to give unpredictable results (Abranches *et al.*, 1991), although recent advances in manipulating cultures to produce almost pure populations of metacyclic promastigotes (Bates & Tetley, 1993) may provide parasites which are more infectious than those of conventional cultures. Amastigotes (Lanotte *et al.*, 1979; Keenan *et al.*, 1984; Abranches *et al.*, 1991) more consistently infect dogs than metacyclic promastigotes from stationary phase cultures but, it may be argued, amastigotes are not the forms of the parasite against which dogs need to be protected by vaccination. Moreover, inocula are extraordinarily high (e.g. 10^8 amastigotes per kg. body weight) (Abranches *et al.*, 1991) which, as Vidor *et al.* (1988) noted, must be several orders of magnitude higher than the numbers of promastigotes deposited by the bite of an infected fly.

Faced with the problem of how to infect dogs experimentally, we chose the intradermal inoculation of promastigotes harvested from the midguts of colonized sandflies infected by feeding on a leishmanial dog. The clinical and immunological responses of a sample of the inoculated dogs were recently described by Pinelli *et al.* (1994). In the present paper, we give details of how we prepared the infective doses and report the results of inoculating 25 dogs. The methods are given in sufficient detail for the procedure to be repeated. We conclude that this challenge is the best at present available, although rearing large numbers of sandflies, infecting them, and harvesting the promastigotes demand levels of expertise which are not readily available.

MATERIALS AND METHODS

SANDFLIES. Two laboratory colonies of *Phlebotomus perniciosus* Newstead were used. The first generation reared from flies collected on the island of Gozo, Malta, was used for a test feed on a leishmanial dog. The second generation bred from females caught near Murcia, Spain, was used in the experiment to infect dogs. The sandflies were reared at Ascot by feeding the females on rabbits and the larvae on an aerobically composted mixture of equal parts of rabbit faeces and rabbit pellets (Killick-Kendrick & Killick-Kendrick, 1987; Lawyer *et al.*, 1991). For the experiment, they were taken to Madrid as pupae and kept until adults emerged.

DOGS. The dogs were all beagles accommodated at the Barajas (Madrid) kennels of the Llorente Institute. Before the experiment, they were vaccinated against distemper, hepatitis, leptospirosis and arboviruses. Twenty-six were used for the experiment and 19 were kept as control, uninfected dogs.

PARASITE. Sandflies were infected with *Leishmania infantum* (Laveran & Mesnil) by permitting them to feed on a dog from the environs of Madrid with a natural infection. The code number of the isolate is MCAN/ES/88/ISS441 DOBA.

INFECTION OF SANDFLIES. The dog was tranquilized with 1.6 ml Combelen (Bayer) given intramuscularly. To confirm that a high proportion of sandflies would become infected when fed on the dog, a test was made with the Maltese flies which were offerred a bloodmeal by putting the head of the dog inside a large gauze cage (42 cm^3) of flies for 55 mins. On the following day, fed females were transferred to a small gauze cage (14 cm^3) that was enveloped in a closed plastic bag containing a damp piece of absorbent cotton wool. The flies were maintained on 50 % sucrose at 28°C for seven days when their midguts were dissected and examined for promastigotes. Of 17 females examined, 16 (93.8 %) were infected confirming that the dog was suitable for infecting flies for the experiment and that the anaesthetic (Combelen) had no noticeable effect on the development of the parasite in the fly.

Eight days after the test feed, the Spanish *P. perniciosus* were offered an infecting bloodmeal. To ensure a long period of anaesthesia, the dog was given an initial dose of 2 ml Combelen i.m. supplemented 50 mins later by a second dose of 1 ml i.m. The head of the dog was kept for 2h 30 mins in a large cage containing hundreds of flies. The cage was then enclosed in a plastic bag and left overnight at room temperature (22-23°C).

On the following day, all fed females in the cage were tubed individually and transferred to six small cages each of which was again enveloped in a plastic bag containing damp cotton wool. The flies were given 50 % sucrose, changed daily, and were maintained at 26-27°C. Flies remaining in the large cage were killed and counted. Before the bloodmeal, the cage had contained 1,636 flies of which 939 (57 %) were females. Of these, 533 (58 %) had engorged.

PREPARATION OF A SUSPENSION OF PROMASTIGOTES. Nine days after the sandflies had engorged, a test was made to see how well promastigotes from the midguts of 14 flies survived in sterile phosphate bufferred saline, pH 7.2 (PBS), kept cool with crushed ice. The parasites were still actively motile on the following day.

Ten days after the infecting feed, infective doses of promastigotes were prepared for 26 dogs and four control hamsters at an arbitrarily decided rate of 10

flies per animal, plus 10 in case of wastage, i.e. 310 female flies (plus four later to replace flies spoilt during dissection). The flies were caught with mouth aspirators in batches of 15 and narcotized with CO_2. They were washed in a dish of PBS containing just enough detergent for them to sink. After two rinses in PBS, they were grouped in batches of five in small glass Petri dishes of PBS kept chilled by standing on ice. Dissections of the midguts of the flies were done by three experienced dissectors who were given the washed flies five at a time. The guts were dissected out in cold PBS and then transferred to three small tissue grinders standing in crushed ice. Each gut was broken as it was put in the grinder. The time from the first flies being killed until the end of the dissection was 6 h 20 mn, which included one break of 25 mn. The average time to dissect one fly was three and a half minutes and the three dissectors dissected at a collective rate of about 50 flies/h.

PREPARATION OF A SUSPENSION OF SANDFLY SALIVA. A suspension of sandfly saliva was prepared according to the method of Titus and Ribeiro (1988). Salivary glands of 22 female *P. perniciosus* were dissected out and added to 20 µl of 1 % bovine serum albumin in water at pH 7.0. They were then frozen in liquid N_2.

CALCULATIONS OF HARVEST AND PREPARATION OF DOSES. The dissected midguts were pooled and the suspension was gently ground and made up to 2 ml. Counts of promastigotes were made both in a haemocytometer and by the microbead method of Cenini *et al.* (1989). The proportion of morphologically recognizable metacyclic promastigotes (Killick-Kendrick, 1986) was estimated by the examination of smears which were allowed to dry then fixed in methanol and stained in Giemsa's stain.

After the promastigotes had been counted, the suspension was diluted to 8.0 ml with cold PBS. It was then divided into two equal lots, to one of which sandfly saliva was added. The previously prepared frozen suspension of salivary glands was thawed, 2 µl of PBS was added and the solution was mixed by mechanical agitation. 20 µl of this solution was added to 4 ml of the suspension of promastigotes giving a concentration of saliva equivalent to half a gland per dose of 0.1 ml.

INOCULATION OF DOGS. Twenty-six dogs were given two intradermal injections, each of 0.1 ml, in the inner surfaces of each thigh. One dose contained promastigotes alone whereas the other contained promastigotes and the saliva suspension. The suspensions were kept cool with crushed ice throughout the inoculations which took 1h 20 mn to complete.

INOCULATIONS OF HAMSTERS. About two and a half hours after the last dog was inoculated, four young hamsters were injected intradermally with the same doses as the dogs. The animals were regularly weighed until their weights began to fall. They were then killed and their viscera were examined for amastigotes by culture and imprints stained in Giemsa's stain after fixation in methanol.

EXAMINATION OF DOGS. One dog died accidentally. The other 25 dogs were regularly examined for clinical signs of leishmaniasis. Dogs which developed advanced signs were killed for humanitarian reasons. Sera of the dogs were periodically tested for leishmanial antibodies by the indirect fluorescent antibody test (IFAT) (see Pinelli *et al.*, 1994). Titres >1/80 were considered positive. All dogs were subjected to one or more attempts to isolate leishmaniae by the culture in NNN medium of bone marrow, spleen juice or tissue from a popliteal lymph node. Three years after the intradermal inoculation of promastigotes, samples of the dogs were examined with a range of immunological tests to assess their cell mediated immune responses (Pinelli *et al.*, 1994; Pinelli, unpublished observations).

RESULTS

PROPORTION OF SANDFLIES INFECTED. Fifty-three (94.6 %) of 56 sandflies dissected and examined 3-9 days after engorging on the leishmanial dog were infected with promastigotes.

HARVEST OF PROMASTIGOTES. Estimates by two counting methods of the numbers of promastigotes harvested from 320 sandflies are shown in Table I. The figures from the haemocytometer count (3×10^6) are lower than those from the microbead method (4.72×10^6). From these figures and the proportion of metacyclic promastigotes seen in stained smears (7 %), it is estimated that each dog and hamster was inoculated with 5,250 (haemocytometer) to 8,260 (microbead method) metacyclic promastigotes, half of which were in a suspension containing sandfly saliva of half a gland.

INFECTIONS IN CONTROL HAMSTERS. All four hamsters inoculated with the same doses of promastigotes as the dogs became infected confirming that the promastigotes, kept cool on crushed ice, retained their infectivity until 2h 25mn after the last dog had been inoculated.

DOGS. One control dog developed signs of leishmaniasis before the experimental dogs were inoculated. It is assumed to have been infected before it was bought. The kennels were sprayed regularly with insecticides and the remaining 18 control dogs remained clinically, parasitologically and serologically negative. The results of observations on 25 inoculated dogs are summarized in table II.

	haemocytometer	microbead method
Total promastigotes harvested	3×10^6	4.72×10^6
Mean promastigotes harvested per fly	9,677	15,226
Mean metacyclics harvested per fly	677	1065
Parasites per dose* of 0.1 mls.	37,500	59,000
Parasites in two doses*	75,000	118,000
Infective forms per dog* (7 % metacyclics)	5,250	8,260

Table I. – Estimates by two different methods of numbers of *L. infantum* promastigotes harvested from 310 experimentally infected *P. perniciosus*
* After dilution to 8 mls.

group	dog n°	parasite isolated	clinical signs	reciprocals of IFAT titres 89	90	91	92	93	CMIR[a]	after 5 years[b]	category
1	06	yes	yes	40	160	160	320	640	n.e.	killed	susceptible
	08	yes	yes	40	160	160	320	640	n.e.	killed	susceptible
	14	yes	yes	80	>640	640			n.e.	killed	susceptible
	16[c]	yes	yes	40	80	80	160	640	+	killed	susceptible
	17	yes	yes	40	>640	>640	80		-	killed	susceptible
	19	yes	yes	80	640	>640	80		-	killed	susceptible
	21	yes	yes	40	80	320			-	killed	susceptible
	22	yes	yes	40	160	320	640		-	killed	susceptible
	26	yes	yes[d]	40	80	20	80	640	-	oligosymptomatic	susceptible
	31	yes	yes	40	640	>640	640	320	-	killed	susceptible
	33	yes	yes	160	640	>640			n.e.	killed	susceptible
	37	yes	yes	40	640	>640			n.e.	killed	susceptible
2	02	yes	no	40	80	<10	<10	<10	+	healthy	resistant
	11	yes	no	20	80	10	<10	40	n.e.	healthy	resistant
	13	yes	no	>640	>640	640	80	40	n.e.	healthy	resistant
	20	yes	no	20	80	10	<10	<10	+	healthy	resistant
	28	yes	no	80	80	10	<10	<10	+	healthy	resistant
	30	yes	no	80	80	10	80	20	+	healthy	resistant
	35	yes	no	80	80	10	<10	<10	+	healthy	resistant
	43	yes	no	40	80	20	n.e.	640	n.e.	healthy	resistant
	44	yes	no	40	640	10	<10	<10	+	healthy	resistant
3	12	no	no	20	40	10	<10	<10	+	healthy	resistant
	23	no	no	40	40	<10	<10	<10	+	healthy	resistant
	29	no	no	160	80	10	<10	<10	n.e.	healthy	resistant
	41	no	no	40	80	10	40	40	n.e.	healthy	resistant

Table II. – Parasitological, clinical and immunological observations on 25 dogs experimentally infected by intradermal inoculations of *L. infantum* promastigotes harvested from the midguts of experimentally infected *P. perniciosus*.
[a]Cell mediated immune response (Pinelli *et al.*, 1994; Pinelli, unpublished observations) (n.e. = not examined).
[b]Dogs were killed when they developed severe clinical signs.
[c]At first developed a CMIR then, 3 years after inoculation, clinical signs appeared and it was killed 19 months later.
[d]First clinical signs 5 years after inoculation.

SITES OF INOCULATION. All dogs developed leishmanial-like lesions at the sites of injection of promastigotes, both with and without added sandfly saliva. The lesions were typically about 1 cm in diameter, crusted, red and dry, with a swelling of the surrounding skin. They appeared 6-14 weeks after inoculation and healed spontaneously three to four months later. No attempt was made to demonstrate parasites and the lesions were left undisturbed.

ISOLATION OF PARASITES FROM DOGS. Parasites were isolated from the spleen, popliteal lymph nodes or bone marrow of 21 (84 %) of the 25 experimental dogs.

CLINICAL SIGNS OF LEISHMANIOSIS. Twelve (48 %) dogs developed clinical signs of the disease.

SEROLOGICAL RESULTS. Sixteen (64 %) dogs developed positive IFAT titres (>1/80).

CELL-MEDIATED IMMUNE RESPONSES. Three to four years after inoculation, 15 of the dogs were examined with a range of immunological parameters (Pinelli *et al.*, 1994; Pinelli, unpublished observations). Peripheral blood mononuclear cells of eight dogs with no clinical signs exhibited proliferative responses to leishmanial antigens, whereas the cells of six dogs with clinical signs did not. One dog (n° 16) was unusual in that it at first developed a cell mediated immune response but later developed clinical signs and was put down after a further 19 months.

COMBINED RESULTS (table II). Of the 21 dogs from which parasites were isolated, 12 had clinical signs and a positive IFAT (Group 1 in table II). Eleven of the 12 progressed to severe signs and were killed.

Nine other dogs (Group 2 in table II) were parasitologically positive but had no clinical signs and were still healthy after five years. Six of the nine were shown to have had a cell mediated immune response corresponding to that of "asymptomatic-resistant" dogs of Pinelli *et al.* (1994). One of the nine (n° 13) had a high IFAT titre for three years which then fell to negative levels. Another (n° 44) had a raised IFAT titre in the second year which then fell, and a third (n° 43) had an antibody titre of 1/640 in the fifth year but no signs of disease; no assessment was made of its cell mediated immune response.

None of four dogs from which parasites were not isolated (Group 3 in table II) had clinical signs. Evidence that two of them (n^{os} 12, 23) received infective doses of promastigotes is that both developed a cell mediated immune response (Pinelli *et al.*, 1994). This response of the other two dogs in this group was not examined, but one (n° 29) initially had a titre of leishmanial antibodies of 1/160 that, in the following year, fell below the cut-off point. The only evidence that the last dog (n° 41) was infected is that chancres arose at the sites of inoculation. Its IFAT titre never exceeded 1/80; it was not one of the sample examined for a cell mediated immune response.

With two exceptions (n^{os} 16 and 26 in Group 1), the dogs can be classified into susceptible and resistant dogs (table II). Susceptible dogs had demonstrable parasites, clinical signs and persistent circulating antibodies; six out of seven examined were shown not to have developed a cell mediated immune response. One, however, (n° 16) had a cell mediated immune response which later failed to protect it, and another (n° 26) had a negative cell mediated immune response but no clinical signs until five years after inoculation. Thirteen resistant dogs, from nine of which parasites were isolated, had no clinical signs and, with three exceptions (n^{os} 13, 43, 44), had negative levels of antibodies; all of eight examined had demonstrable cell mediated immune responses.

DISCUSSION

Evidence that 21 of the dogs became infected after inoculation of an estimated 5-8,000 metacyclic promastigotes is that parasites were isolated from them. Two of the remaining four from which parasites were not isolated, and which showed no clinical signs of leishmaniasis, are presumed to have been infected because they had chancres at the sites of inoculation and developed a cell mediated immune response. A third dog had a titre of leishmanial antibodies of 1/160 for one year. Evidence for the infection of the fourth dog is the appearance of chancres at the sites of inoculation. We conclude that all 25 dogs were infected by the inoculation of promastigotes harvested from midguts of sandflies. Since the course of infection in the dogs was variable in spite of being given standard doses, we assume that the outcome was determined genetically rather than by the number of parasites inoculated.

The responses of the inoculated dogs mimic nature in several respects. The chancres at the sites of inoculation were like skin lesions in a cohort of 50 dogs introduced into the Cévennes, an endemic area of canine leishmaniasis in France, and kept under observation for two years by Vidor *et al.* (1991). These lesions were in places commonly bitten by the local vector (*Phlebotomus ariasi* Tonnoir) and were undoubtedly at the sites of bites. Of the 50 dogs, chancres were seen in 29, and *L. infantum* was cultured from the lesions of 15 of 23 dogs examined. Twenty dogs with chancres seroconverted and nine remained serologically negative during the period of observation.

In an experimental transmission of *L. infantum* to a dog by the bite of a single sandfly, Rioux *et al.* (1979) recorded a delay of 16 months before the first clinical signs were evident. Most of the incubation periods of the dogs in the present study were 18-24 months and thus of the same order. Another similarity with the natural history of the disease is that, in nature, dogs with neither signs of leishmaniasis nor circulating antibodies have been found with a cell mediated immune response (Cabral *et al.*, 1992; Pinelli *et al.*, 1994).

The dogs were inoculated intradermally because this was the closest to putting the promastigotes in the place they would be deposited by a biting sandfly. It is known that Langerhans' cells migrate from the epidermis to the dermis and transport promastigotes from there to draining lymph nodes (see review by Moll, 1993). This may be an essential step in presenting antigens and, if the individual has the capability, of initiating a cell-mediated immune response. Intradermal inoculations of metacyclic promastigotes are clearly more natural than intravenous, intraperitoneal or intrahepatic inoculations of many millions of amastigotes (Lanotte *et al.*, 1979; Keenan *et al.*, Abranches *et al.*, 1991).

We added sandfly saliva to half of the inoculum because Titus and Ribeiro (1988) showed that the development of cultured promastigotes of *L. major* Yakimoff & Schokhor in the skin of mice was enhanced by the addition of the saliva of *Lutzomyia longipalpis* (Lutz & Neiva) (see also Theodos *et al.*, 1991; Samuelson, 1991). However, we have no evidence to suggest that the infectivity of the promastigotes harvested from midguts was affected by the addition of sandfly saliva. Chancres appeared at sites of inoculation of promastigotes with and without added saliva. This may be because the suspension of promastigotes to which no saliva was added nevertheless contained some saliva released from glands escaping from the thorax when the head was cut off before drawing out the midgut.

Although the promastigotes harvested from experimentally infected sandflies were highly infective to the dogs, infecting large numbers of sandflies and harvesting the parasites demand high levels of expertise. Several aspects of the methods we used are worthy of note. Firstly, there are no difficulties in rearing hundreds of *P. perniciosus* which is an easy sandfly to breed in the laboratory [see references in Killick-Kendrick *et al.* (1991), also Ready & Croset (1977) and Molina (1991)]. We used the second generation of more than 1500 flies of which nearly 1000 were females. Secondly, dogs with leishmaniasis infect widely different proportions of sandflies (Rioux *et al.*, 1972; Gradoni *et al.*, 1987; Molina *et al.*, 1994) and it is necessary to make preliminary tests to select a suitable dog. Thirdly, mortality of engorged sandflies is minimized if they are left unhandled for 24hrs. Survival thereafter is good providing the flies are kept at a correct temperature and high relative humidity, and are given fresh sucrose solution daily. This sugar solution should be concentrated to minimize bacterial and fungal contamination. Fourthly, the dissection of the midguts to harvest the promastigotes was slow, even for experienced dissectors, because all the flies were gravid. Although dissections would have been easier if females had been permitted to lay their eggs, this was not feasible because, in the laboratory, most of the flies would undoubtedly have died at, or shortly after, oviposition. Lastly, seven hours elapsed between the dissection of the last sandfly and the inoculation of the hamsters, all of which became infected. Throughout this time, the suspensions of promastigotes, at pH 7.2, were kept chilled with ice. These conditions appear to have been adequate for the survival of the metacyclic promastigotes.

Future studies should be aimed at preparing cryopreserved stabilates of metacyclic promastigotes of known infectivity, titrated in hamsters. This would provide standardised doses of parasites to challenge dogs immunised with putative vaccines. Technically, it would be simpler to work with cultured promastigotes than with promastigotes harvested from experimentally infected sandflies. An essential prerequisite is to determine whether or not sandfly saliva enhances the infectivity of cultured metacyclic promastigotes of *L. infantum*.

In devising a mathematical model of canine leishmaniasis, Hasibeder *et al.* (1992) postulated the existence of two kinds of dogs, type A and type B. When exposed to infection by *L. infantum*, type A dogs were defined as initially without clinical signs but serologically positive, eventually developing signs and becoming infectious to sandflies. Type B dogs were said to be animals which, although never infectious to sandflies and with few or no clinical signs, also become serologically positive but self cure. Our results broadly support this grouping, although demonstrable antibodies in type B dogs may be ephemeral and, unless the dog is kept under constant observation, may not be detected (Dye *et al.*, 1993). Recent observations suggest that the assumption by Hasibeder *et al.* (1992) that type B dogs never infect sandflies is no longer tenable. Molina *et al.* (1994) showed that some dogs with no clinical signs of leishmaniasis can infect sandflies, a finding which is not only important in modelling the disease but also questions the value of attempting to control human visceral leishmaniasis caused by *L. infantum* (or "*L. chagasi*") by surveys of dogs followed by the des-

truction of those assumed to be infectious to sandflies. In the present work, parasites were isolated from nine of 13 dogs classified as resistant (= type B) (Group 2 in table II), but no attempt was made to see if they were infectious to sandflies.

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