Establishment of asymptomatic *Leishmania donovani* infection in Indian langurs (*Presbytis entellus*) through intradermal route*

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Received 14 August 2000; revised 23 November 2001

Indian langurs, which were previously reported to be highly susceptible, were infected intradermally using variable numbers of promastigotes along with different doses, 1/2 pair, 5 pairs and 10 pairs respectively of salivary gland lysate (SGL). Although, all the monkeys developed mild infection and remained subclinically infected throughout the observation period, which later resolved, none of them could develop the classical disease. No marked antigen specific antibody or lymphoproliferative response was noticed throughout the experimental period. However, a late IFN-γ response (by day 90 p.i.) was demonstrated in monkeys infected with 2x10⁶ promastigotes +10 pairs SGL. It seems that a single intradermal dose of promastigotes with or without SGLs had a "vaccine" like effect. Perhaps, multiple frequent inoculations, as happens in the natural situation, may be necessary for the development of full-blown disease.

Visceral leishmaniasis (VL), a potentially fatal disease in humans, is transmitted by sandfly of *Phlebotomus papatasii*. During its blood meal sandfly salivates few hundreds of metacyclic promastigotes form of the parasite into the skin of vertebrate host. This is followed by establishment of infection, either as classical Kala-azar, or less severe self-limiting disease.

In experimental conditions, Indian langur monkeys (Presbytis entellus) developed classical disease symptoms by iv inoculation of 1x10⁶ *Leishmania donovani* amastigotes. The present study has been aimed at establishing disease in susceptible langur monkeys through intradermal route along with salivary gland lysates (SGL) (as occur in natural infection) and to study the immune response following infection.

Young male langur monkeys (approximately 3-5 kg body weight) were caught in the wild and reared as described elsewhere. Care, management, experimental protocols and number of animals in each group were according to ethical guidelines laid down at CDRI. Parasitic and immunological assessments (antibody estimations, lymphocyte proliferation and cytokine (IFN-γ and IL-2 by using Genzyme kit) assay were done at variable times throughout the experiments.

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*Intradermal inoculation of L. donovani with 1/2 pair SGL of Phlebotomus papatasii—Monkeys (8) were infected intradermally with 100x10⁶ promastigotes of L. donovani (Dd8) plus 1/2 pair SGL of *P. papatasii* (gifted by Dr. David Sacks, NIH, USA) on right forearm and similarly 4 animals were given promastigotes alone. The former group developed low-level infection by day 60 p.i. which subsequently remained low till the observation period of 230 p.i. (Fig. 1A); while the control group did not develop any parasite. The antigen specific antibody levels showed a gradual increase by day 30 p.i. in monkeys infected with promastigotes along with SGL which almost persisted till day 180 p.i., whereas the rise in antibody level in the monkeys infected with promastigotes alone was less marked (Fig. 1A) (cut-off point for pooled negative sera is 0.22 ± 0.07 and for pooled positive sera is 1.32 ± 0.14 at the sera concentration of 1:100). There was no mitogen and antigen specific LTT response in both the groups throughout the experiment (unpublished data).

*Intradermal inoculation of L. donovani with 5 pairs SGL of P. papatasii—As in earlier experiment infection could not be established, in another batch of three monkeys an enhanced dose of 5 pairs of SGL of *P. papatasii* with 100x10⁶ promastigotes intradermally was given on the right forearm while in two the same...*
number of parasites were given alone by the same route to serve as control. There was no change in parasite burden in monkeys of both the groups (Fig. 1B). The antibody profile in both groups of monkeys was similar to those as observed in above experiment (Fig. 1B). The only difference was that peak O.D. value was observed on day 90 p.i. Mitogen (unpublished data) and antigen specific LTT responses were absent in both the groups throughout the experiment. However, very low levels of IFN-γ and IL-2 were detected in these animals (Table 1A).

**Intradermal inoculation of *L. donovani* with 10 pairs SGL of *P. papatasi* and *P. duboscqui*—Since infection could not be established with previous experiments, a further attempt was made at establishing infection by enhancing the dose of SGL to 10 pairs. This time SGLs were used from two sandfly species namely *P. papatasi* and *P. duboscqui* (gifted by Dr. C.O. Anjili, KEMRI, Kenya). Out of 13 monkeys, 4 were inoculated with $2 \times 10^6$ promastigotes and 10 pairs of SGL of *P. papatasi*, 6 with $2 \times 10^6$ promastigotes and 10 pairs of SGL of *P. duboscqui* (5 pairs on each side of upper eyelid) while the rest three inoculated with $2 \times 10^6$ promastigotes without SGL served as control.

Moderate parasite burden could be seen in all the experimental monkeys by day 45 p.i. (Fig. 1C). However, they failed to develop any evidence of disease and the infection resolved by day 150 p.i. A sharp rise in antibody level was observed as early as day 15 p.i. in all the three groups of monkeys which eventually decreased corresponding to the parasite burden of their respective groups and reached the normal value by day 150 p.i. (Fig. 1C). The rise in antibody level was more marked in the group infected with SGL of *Phlebotomus duboscqui* rather than *Phlebotomus papatasi* which remained higher throughout the observation period. The mitogen specific (unpublished data) and antigen specific LTT response was not demonstrable in any of the three experimental groups throughout experimental period (Table 1B). IFN-γ and IL-2 levels in this set of experiment was higher by day 45 p.i. as compared to the former one infected with five pairs lysate and subsequently increased by day 90 p.i. No statistical analysis could be applied to these data, as the number of animals in each group was restricted by ethical considerations.

In an attempt to mimic the biology of natural transmission, we have tried to establish a model of visceral leishmaniasis in a non-human primate species by intradermal inoculation of variable numbers of *L. donovani* metacyclic promastigotes along with SGLs from natural vectors, *P. papatasi* and *P. duboscqui*. In nature, sandflies salivate approximately 10-1000 promastigotes during each bite into the skin along with the promastigotes. The inclusion of sandfly SGL in the infecting inoculum has been reported to enhance infectivity of leishmania parasites irrespective of the vector parasite combination. SGL has recently been shown to deviate the immune response to
Needle inoculation of variable doses of *L. donovani* promastigotes with or without SGL to a number of monkeys failed to establish classical disease symptoms. The parasite load was higher in the batch, which received 10 pairs SGLs plus 2×10⁶ promastigotes as compared to the other groups receiving 1/2 or 5 pairs SGLs alongwith 100x10⁶ promastigotes. In spite of the parasite dose being 50 times less, low level asymptomatic infection was established irrespective of site of injection being either in the fore arm or upper eyelid.

Thus, it appears that the dose and route of inoculation do induce low-level cellular response of adequate strength to prevent unrestricted parasite multiplication and disease development. The lower dose of parasite (2×10⁶) has been less effective in this resulting in higher parasite load than in animals given the higher parasite load (100x10⁶). The animals of all the groups developed low level of specific antibody responses and remained subclinically infected in contrast to the monkeys with active disease (by i.v.route). The failure to develop a parasite specific lymphocyte proliferation in monkeys infected with promastigotes with or without 1/2 (unpublished data) and 5 pairs of SGL indicated that the asymptomatic infection established in these animals is inadequate to stimulate a measurable cellular response. However, the increase in IFN-γ and IL-2 response at day 90 p.i. in the group infected with 10 pairs of SGL doses indicate that these animals probably have developed a measurable cellular response. Similar observations has been made by Gicheru et al. with *L. donovani*-vervet monkey system where animals remain subclinically infected. They have also shown high levels of IFN-γ when PBMCs were stimulated with parasite antigen.

Hence, a low level asymptomatic parasite infection could be established in Indian langurs by i.d. inoculation of promastigote along with SGL. It seems that a single intradermal dose of promastigotes with or without SGLs had a "vaccine" like effect. Perhaps, multiple frequent inoculations, as may be happening in the natural situation, may be necessary and these should be at short intervals so that there is not enough time for protective immunity against the parasite to develop. Future attempts should be made to establish the disease model with multiple small inoculations of the parasite as well as by infected sandfly bites.

The technical assistance by Mr. S.C. Bhar is gratefully acknowledged. AM and PS are thankful to W.H.O., Geneva and CSIR, New Delhi for financial assistance. JKS is thankful to Government of India for his placement in the Scientist Pool Scheme. This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR).

### References


