sequently, one can propose that forskolin treatment leads to an increase in cyclic AMP, activation of cyclic AMP–dependent protein kinase, phosphorylation of MLCK, dephosphorylation of myosin by phosphatases, and relaxation of tracheal smooth muscle.

However, an increase in cyclic AMP has been associated with a decrease in intracellular Ca^{2+} in smooth muscle (*13*). Since MLCK requires calcium-calmodulin for activity (*14*), relaxation could be due to inactivation of MLCK after a decrease in intracellular Ca^{2+}. Nevertheless, Kerrick and Hoar (*15*) have relaxed skinned smooth muscles contracted with high concentrations of calcium by adding the catalytic subunit of cyclic AMP–dependent protein kinase. This experiment suggests that it is possible to relax smooth muscles, presumably by a mechanism that involves MLCK phosphorylation, even in the presence of high intracellular calcium.

Finally, the data indicate that myosin is dephosphorylated when methacholine-contracted muscles are relaxed with atropine or forskolin (Table 1). These agents work by different mechanisms (*7*), and the fact that myosin is dephosphorylated after the addition of either agent suggests that myosin dephosphorylation may be essential for relaxation of smooth muscles.

In conclusion, these results show that MLCK is phosphorylated in intact tracheal smooth muscle, that MLCK phosphorylation increases when tracheal muscles are treated with forskolin, and that myosin dephosphorylation may be essential for relaxation of previously contracted muscles. Thus MLCK phosphorylation and myosin dephosphorylation appear to be part of the mechanism by which cyclic AMP causes relaxation of intact smooth muscles.

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Identification of an Infective Stage of *Leishmania* Promastigotes

Abstract. *Sequential development of* Leishmania *promastigotes from a noninfective to an infective stage was demonstrated for promastigotes growing in culture and in the sandfly vector. The generation of an infective stage was found to be growth cycle–dependent and restricted to nondividing organisms.*

Despite the importance of leishmaniasis among the parasitic diseases of man, our understanding of some elementary aspects of the developmental cycle of *Leishmania* parasites remains incomplete. Most *Leishmania* species are known to multiply as intracellular amastigotes in macrophages of their vertebrate host and as extracellular promastigotes in the midgut of their sandfly vector (*1*). A basic question remaining is whether or not sandfly promastigotes differentiate into an infective stage. If there is an infective stage of *Leishmania* in invertebrates, then, unlike other hemoflagellates, it does not appear to have a readily distinguishable morphological identity. Morphological differences between dividing midgut forms and those found anteriorly have been described (*2*); however, to our knowledge there has been no evidence that these changes reflect development of promastigotes into an infective stage. There is some

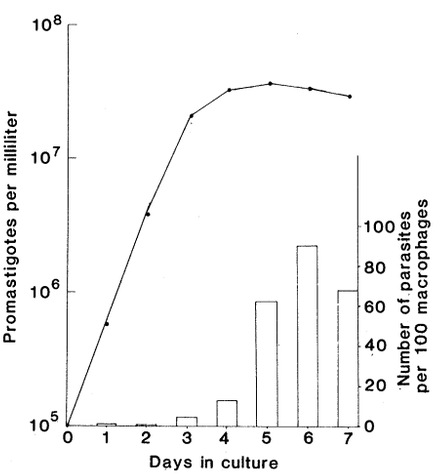

Fig. 1. Infectivity of cultured promastigotes, taken from various points in their growth curve, in mouse peritoneal macrophages in vitro. *Leishmania tropica* promastigotes (Friedlin strain NIH) were obtained from logarithmic phase cultures and inoculated (10^5 promastigotes per milliliter) into fresh medium containing medium 199 with Earle's balanced salts, 20 percent heat-inactivated fetal calf serum, 12 m*M* Hepes, 20 m*M* L-glutamine, and penicillin-streptomycin (50 μg/ml). One to seven days after initiation of the cultures, promastigotes were washed twice in medium 199, adjusted to a concentration of 5×10^6 promastigotes per milliliter, and used to infect adherent cultures of mouse peritoneal macrophages. Peritoneal cells were obtained from C57BL/6 mice (Jackson Laboratory), washed twice, and resuspended (2.5×10^6 cells per milliliter) in RPMI 1640 medium containing 10 percent fetal calf serum, 12 m*M* Hepes, 20 m*M* L-glutamine, and penicillin-streptomycin (50 μg/ml). Cells were plated in 0.4 ml on eight-chamber Lab-Tek tissue culture slides (Miles Laboratories) and allowed to adhere to the slides overnight at 37°C in 5 percent CO_2. Nonadherent cells were removed; adherent cells were infected with 10^6 promastigotes at an approximate ratio of two parasites per macrophage. After a 2-hour infection period at 35°C, free promastigotes were removed by repeated washings and cultures were incubated for up to 7 days at 35°C in 5 percent CO_2. Slides were fixed in absolute methanol for 30 minutes and stained with Diff-Quick solutions to make the intracellular amastigotes visible. For each culture over 500 cells were counted to determine the percentage of macrophages infected and the number of parasites per cell. The bars represent the total number of amastigotes per 100 macrophages present 3 days after infection with promastigotes obtained from the corresponding points in their growth curve.

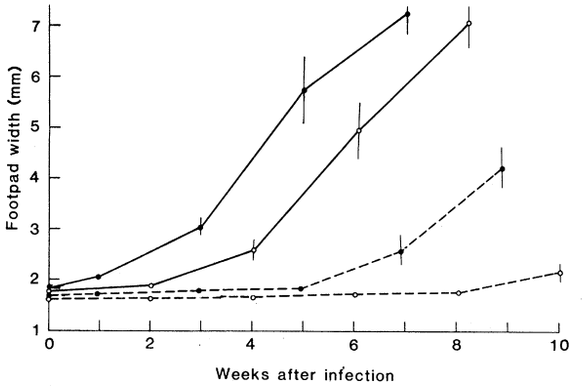

Fig. 2 (left). Infectivity of logarithmic (dashed lines) and stationary (continuous lines) phase promastigotes for BALB/c mice. *Leishmania tropica* Friedlin promastigotes were passaged weekly in promastigote medium, and parasites obtained from mid-logarithmic and late stationary cultures were used to infect the footpads of BALB/c mice (Jackson Laboratory). Promastigotes were washed twice in medium 199 and 10^6 (●) or 10^4 (○) parasites were inoculated subcutaneously into the left hind footpad. Footpads were measured weekly with a direct-reading vernier caliper. Values are means ± standard deviations for five to six mice per group.

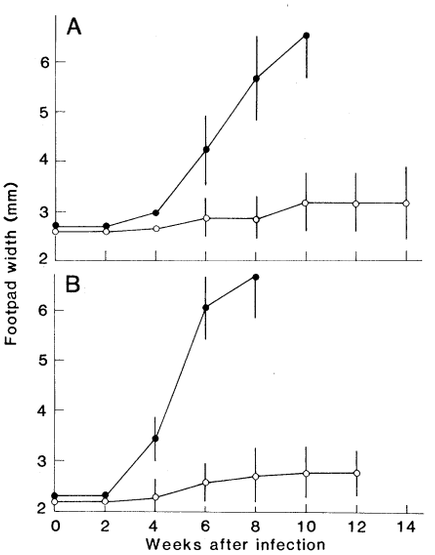

Fig. 3 (right). Infectivity of promastigotes taken from fly midguts in BALB/c mice. *Lutzomyia anthophora* flies reared as described by Endris *et al.* (*8*) were allowed to feed on footpad lesions of BALB/c mice infected with *L. tropica* Friedlin. Three days or 7 to 10 days later the flies were individually dissected in medium 199. The head was removed and discarded and the entire digestive tract was teased from the posterior segment and examined under dark-field illumination (100×) without a cover slip. Midguts positive for promastigotes were ruptured to release active parasites. Promastigotes from each infection time point were pooled, counted, washed once in medium 199, and inoculated into BALB/c mouse footpads. In (A) each mouse was inoculated with 2500 promastigotes pooled from four flies with 3-day-old infections (○) or from eight flies with 7- to 8-day-old infections (●). In (B) each mouse was infected with 10^4 promastigotes pooled from seven flies on day 3 of infection (○) or from eight flies on days 7 to 10 (●). Footpads were measured weekly with a direct-reading vernier caliper. Values are means ± standard deviations for five to six mice per group.

suggestion that differentiation of promastigotes can occur in vitro; several investigators have found a correlation between the age of *Leishmania donovani* promastigotes in culture and their infectivity for experimental animals (*3*). The implications of these findings have not been generally appreciated, since cultured promastigotes, which are the most convenient source of *Leishmania* parasites for study, are still considered biologically uniform populations with respect to infectivity.

We now report that the infectivity of *Leishmania* promastigotes taken from stationary cultures greatly exceeds that of promastigotes from cultures in the logarithmic phase. More important, we found that identical developmental changes occurred during growth of promastigotes in the fly. These results strongly indicate that *Leishmania* promastigotes develop from a noninfective to an infective stage in the sandfly vector.

Leishmania tropica promastigotes, taken from various points in their growth curve, were used in vitro to infect macrophages normally resident in mouse peritoneum. After an initial 2-hour infection period free promastigotes were removed from the adherent cultures. After 3 days the number of intracellular amastigotes was determined. As shown in Fig. 1, promastigotes taken from logarithmic phase cultures were noninfective. Parasites derived from cultures approaching the stationary phase showed a continuous increase in their ability to establish intracellular infections. The most infective populations were derived from completely stationary cultures. Generation of infective forms in stationary populations of cultured promastigotes is a characteristic of all *Leishmania* species that we have studied. This includes additional isolates of *L. tropica, L. mexicana mexicana, L. mexicana amazonensis, L. braziliensis panamensis*, and *L. braziliensis braziliensis*. We have seen the same pattern of infectivity in vitro with cloned promastigotes of *L. tropica* or *L. mexicana*, which rules out the possibility that the development of infective parasites in stationary populations is due to the selection of genotypically distinct parasites in aging cultures.

The infectivity of logarithmic and stationary promastigotes in vitro was predictive of their virulence in mice. Infection of the footpads of BALB/c mice with 10^6 or 10^4 *L. tropica* Friedlin stationary promastigotes resulted in footpad lesions within 4 weeks. The footpads became greatly enlarged, ulcerated, and necrotic and by 7 to 10 weeks had dropped off in all mice (Fig. 2). After 7 to 10 weeks of infection with 10^6 or 10^4 promastigotes in mid-logarithmic phase, footpad lesions had only begun to appear.

It seems that promastigote populations are not uniform with respect to infectivity. Our findings suggest, rather, that the generation of infective forms is related to the growth cycle and is restricted to nondividing organisms. To determine whether similar developmental events occur during growth in the sandfly, we obtained promastigotes from the midgut of infected *Lutzomyia anthophora*, that had fed 3 days or 7 to 10 days previously on footpad lesions of BALB/c mice inoculated with *L. tropica*. As shown in Fig. 3, promastigotes obtained 3 days after fly infection, when ingested blood was still present, were essentially avirulent in BALB/c mice. In the two experiments represented, only two of five mice (Fig. 3A) and two of six mice (Fig. 3B) had demonstrable lesions, and each mouse with lesions failed to develop signs of progressive disease characteristic of *L. tropica* infections in BALB/c mice. In contrast, midgut promastigotes obtained 7 to 10 days after fly infection were highly virulent, producing uncontrolled growth of the primary lesion and loss of the footpad by 10 to 12 weeks.

Inoculation of promastigotes from flies has repeatedly been shown to initiate infection in susceptible hosts (*1, 4*). Thus it has been generally assumed that promastigotes in the gut of sandflies are invariably infective to a susceptible vertebrate. There were, however, a few early studies in which midgut promastigotes failed to establish infection (*5*). The most interesting of these studies was by Parrot and Donatien (*6*), who were unable to infect mice with promastigotes from the midguts of *Phlebotomus papatasi* with 34- to 48-hour-old infections of *L. tropica*. These results and our own findings suggest that actively dividing promastigotes obtained shortly after fly infection are, like their logarithmic phase counterparts in vitro, essentially avirulent.

The generation of an infective stage in

the midgut by day 7 suggests that digestion and passage of the blood meal creates conditions in the midgut that resemble the nutrient-depleted environment of stationary cultures. Thus the differentiation of promastigotes into an infective stage appears to occur in response to adverse growth conditions. This is consistent with the biology of other protozoa. Encystation of organisms such as *Entamoeba* and *Giardia*, as well as other trypanosomatids such as *Leptomonas* and *Crithidia*, occurs in response to nutrient deficiency and enables these parasites to survive conditions that would kill the dividing, noninfective forms. Transformation of cultured *Trypanosoma cruzi* epimastigotes into infective stage trypomastigotes occurs during the stationary phase (*7*). The avirulence of dividing promastigotes does not prevent effective transmission, since flies usually do not take another meal until the previous meal has been digested and passed. The development of infective promastigotes in nondividing populations coincides with the time at which another meal is sought by the fly. Parasites found in mouthparts are never in active division (*2*). The generation of an infective stage in the midgut before their migration to mouthparts ensures that parasites which are transmitted during feeding by the fly are preadapted to survival in the vertebrate.

In conclusion, *Leishmania* promastigotes sequentially develop into an infective stage both in culture and in the sandfly. The immediate implication of these findings is that investigators using cultured promastigotes must take their potential biological heterogeneity into account. More important, the easy generation of infective and noninfective stages in vitro will enable study of the antigenic, biochemical, and physiological changes that accompany transition of these parasites from invertebrate to vertebrate environments.

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Sequential Interaction of Glia Maturation Factor with Insulin

Abstract. *Astroblasts in culture proliferated when exposed to glia maturation factor for at least 2 hours and then to insulin, but not when exposed in the reverse order. The sequential relation suggests that glia maturation factor is a competence factor.*

Glia maturation factor (GMF) is a potent mitogen as well as maturation agent for astrocytes (*1–5*). When GMF is added to density-arrested cultures of rat astroblasts, the cells proliferate and then undergo chemical differentiation. The mitogenic influence of GMF is evident in both confluent and sparse cultures (*6*). Although the presence of fetal calf serum is necessary for this mitogenicity, serum components can restore part of the effect (*6*). We recently observed (*6*) that the growth rate of astroblasts in the serum-free medium N2 (*7*) supplemented with GMF approaches that in 10 percent serum alone. We now report that the mitogenic effect of GMF and medium N2 can be reproduced if all the ingredients of N2 but insulin are omitted and that the same effect is obtained if the cells are exposed

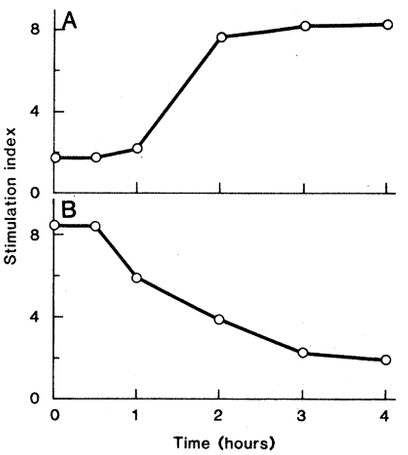

Fig. 1. Time dependence of the establishment and decay of GMF-induced competence. Rat astroblasts were cultured and sequentially stimulated by GMF and insulin as described for condition 7 in the legend to Table 1, except that in (A) the length of exposure to GMF varied and in (B) the cells were exposed to GMF for 2 hours and the interval between the withdrawal of GMF and the beginning of insulin exposure varied. During the interval the cells were cultured in the base medium without serum. Fibronectin was included in all serum-free media. Each value is the mean for quadruplicate samples and has a standard deviation of 5 percent or less.

Table 1. Glia maturation factor as a competence factor for astroblast proliferation. In each experimental condition the base medium was used with various additives. Medium N2 contained insulin (5 μg/ml), transferrin (100 μg/ml), 20 n*M* progesterone, 100 μ*M* putrescine, and 30 n*M* selenium (*7*). When alone or in combination with GMF, insulin was used at a concentration of 5 μg/ml. The GMF, a sample from bovine brain purified 10,000-fold (*6*, *8*), was used at a concentration of 200 ng/ml. In conditions 1 through 6 the base medium and additives were renewed at 2 days. In condition 7 the monolayer culture was stimulated with GMF for 4 hours, washed twice with the base medium, and immediately switched to insulin, which was subsequently renewed at 2 days. In condition 8 the cells were exposed to insulin for 4 hours and then to GMF. The total exposure period for all cultures was 4 days. The stimulation index is the ratio of the final number of cells to the initial number, as determined with a Coulter counter, the initial number being 8.4×10^3 cells per square centimeter. Each value is the mean for quadruplicate samples and has a standard deviation of 5 percent or less. Human serum fibronectin (2 μg/ml) was included in all media lacking fetal calf serum.

Condition	Stimulation index	Condition	Stimulation index
1. No additives	1.55	5. Insulin	1.91
2. Medium N2	1.96	6. GMF	2.26
3. Medium N2 + GMF	9.56	7. First GMF, then insulin	8.95
4. Insulin + GMF	9.41	8. First insulin, then GMF	2.26