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Leishmania amazonensis: Chemotaxic and osmotaxic responses in promastigotes and their probable role in development in the phlebotomine gut

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Abstract

Taxic responses may play a role in development of *Leishmania* in their phlebotomine sand fly vectors. They are possibly responsible for movement of the parasites towards the anterior regions of the gut, from where they would be transmitted to the vertebrate host. A methodology capable to distinguish chemotaxic from osmotaxic responses was described and used to characterise taxic responses in *Leishmania* promastigotes. These were able to respond to chemotaxic as well as to osmotaxic stimuli. Like bacteria, promastigotes were capable to undergo "adaptation," a phenomenon by which they stop responding to a continuos stimulus. A model capable to explain how a relatively small number of different receptors works to perceive gradients in chemotaxic responses was proposed. According to this model, these receptors possess low specificity and a wide range of affinities varying from high to low. A low specificity makes the same receptor able to bind to a large number of different but structurally related molecules and; a wide range of affinities (considering a population of receptors), implies that the number of receptors "occupied" by attractant molecules along a gradient would go growing step by step.

Index Descriptors: Chemotaxis; Osmotaxis; Promastigotes; *Leishmania* development; Phlebotomine sand flies

1. Introduction

During the final phase of their development in phlebotomine sand flies, *Leishmania* promastigotes migrate to the anterior regions of the insect gut, from where they are transmitted to the vertebrate host. Killick-Kendrick (1978) proposed that carbohydrates ingested and stored in the diverticulum formed a gradient along the gut and were responsible for this migration.

Bray (1983), using a crude methodology, was the first to obtain experimental evidence that promastigotes could present taxic responses. Later, we developed a much more sensitive method to assay for responses in *Leishmania* (Oliveira et al., 2000). In this method, a gradient of the substance to be assayed was produced inside a capillary tube, which was submerged in a promastigote suspension. After an incubation period, attraction of the substance tested was measured by counting parasites inside the capillary tube. In this study, eight different carbohydrates were tested and promastigotes attracted by all of them. These responses were interpreted as being chemotaxic.

Leslie et al. (2002) used this capillary tube technique to observe that promastigotes migrated towards any of the substances they used in the tests, including NaCl. These responses appeared to depend solely on an osmotic gradient, thus the taxic responses presented by the promastigotes were re-interpreted by them as being osmotaxic.

The mechanism involved in chemotaxic responses of bacteria is already reasonably well understood. According

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to Macnab and Koshland (1972), bacteria perceive a concentration gradient by means of a temporal mechanism, that is, when in motion they compare the concentration of the attractant in the surrounding microenvironment with that in the area into which they are moving to. Every time that the concentration increases, they tend to remain swimming in that direction. This behavior directs the bacteria to the source of the chemotaxic stimulus along an ascending gradient.

Bacteria may also respond to variations in the osmotic pressure of the medium by a mechanism similar to chemotaxy, known as osmotaxy (Li et al., 1988). In the presence of an osmotic gradient, osmotaxy makes bacteria move towards an ideal osmotic pressure. They are repelled both by excessively low and high concentrations.

Adaptation is a fundamental characteristic of taxic responses in bacteria. This consists of the capacity to stop responding to a stimulus when it becomes constant, that is, when there is no variation in the concentration of the taxic agent or osmolarity of the medium. Adaptation is not instantaneous, a certain time always being needed for it to become established after the final variation occurs. In bacteria, if the concentration of the attractant stops varying within 5 min adaptation occurs and they show an increased frequency of directional change. These constant changes of direction facilitate the casual encounter of a new ascending gradient. As long as this new gradient provides the bacteria with variation in the attractant concentration, they will keep moving in a straight line, towards an ascending gradient (Macnab and Koshland, 1972).

In this study, we developed a methodology based on those of Li et al. (1988) and Macnab and Koshland (1972) that allow the mechanisms involved in the taxic responses of *Leishmania amazonensis* promastigotes to be characterized. Since this method allows chemotaxic responses to be distinguished from osmotaxic ones, we also present evidence that promastigotes, like bacteria, respond to both types of stimuli.

2. Materials and methods

2.1. Parasite

Promastigotes of *L.* (*Leishmania*) *amazonensis* (strain IFLA/BR/1967/PH8) were cultivated in MD26 defined medium (Melo et al., 1985), with added vitamin B12 (0.0064 μg/ml), PABA (0.1256 μg/ml), 5% bovine fetal serum and 22 mM sucrose. Cultures were maintained at 25 °C with intermittent stirring (3 min of stirring followed by 10 min at rest). The experiments were carried out with promastigotes on the sixth day of culture in the stationary phase.

2.2. Measurement of mean time of straight line movement

This methodology was developed to investigate chemotaxic and/or osmotaxic responses of *Leishmania* promastigotes. The mean time that the promastigotes remain moving in a straight line was measured in the absence and presence of a homogenous concentration of the taxic agent under study. Movement in a straight line is defined here as the absence of abrupt changes of direction. Just before an abrupt change in direction, the promastigotes show a movement denominated "tumbling" that is somewhat similar to that described in bacteria. The time of straight line movement (TSLM) counts should be done in the first 15 min after the addition of the taxic agent to the mixture since, they stop responding to a stimulus when it becomes constant. This phenomenon called adaptation occurs within 30–60 min and promastigotes resume behaving like in the control.

Promastigotes were washed three times by centrifugation at 2000*g* at room temperature for 5 min in washing and incubation solution (WIS) (Oliveira et al., 2000). WIS is composed of 30 mM sodium β-glycerolphosphate, 87 mM NaCl, 27 mM KCl, 2 mM $CaCl_2$ and 2 mM $MgCl_2$ at pH 6.8 or 6.0. The final preparation for the TSLM count was obtained by mixing, in a microcentrifuge tube, 250 μl of 2% bovine haemoglobin previously dissolved in WIS, 100 μl of WIS containing the taxic agent in a desirable concentration and 550 μl of WIS. Just before initiating the counting, 100 μl of WIS containing 10^6 washed promastigotes were added to the mixture (final volume = 1 ml). This mixture was maintained in ice bath until use. Haemoglobin (SIGMA code H-2625) was used to minimize adherence of the promastigotes to the glass of the slides and coverslips. Each experiment was performed with a single preparation of WIS to eliminate small differences in concentration of the components of this solution. Aliquots of 10 μl of the final mixture, stored in an ice bath, were used to prepare slides covered by 18 × 18 mm coverslips that were observed under the 400× objective of an optical microscope at room temperature. TSLM was measured using a digital chronometer. According to our experience, the error associated with each measurement is not greater than 1 s. At least 50 counts were made for each concentration of taxic agent or control. A mean of three slides, prepared consecutively, was sufficient for 50 counts. Each slide was observed for up to 5 min by the same person. When Hepes (*N*-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) was used as the taxic agent, the pH was adjusted to 6.8 with Tris (tris(hydroxymethyl)-aminomethane).

2.3. Evaluation of Lutzomyia longipalpis salivary gland extracts as taxic agent for promastigotes

Thirty glandular lobes obtained from 4- to 5-day-old females maintained in a closed colony (Modi and Tesh, 1983), were dissected and transferred to a microcentrifuge tube containing 300 μl WIS. After rupturing the lobes by sonication for 10 s, the tube was centrifuged at 10,000*g* for 5 min. A quantity (250 μl) of the supernatant containing the equivalent of 25 lobes was used to prepare 1 ml of the final TSLM count mixture, as explained in Section 2.2.

2.4. Study of adaptation responses to variations in concentration of the taxic agent

The TSLM method described here is especially useful to investigate adaptation in *Leishmania* promastigotes. TSLM was measured before, immediately after and 30 or 60 min after changes in concentration of the taxic agent. These changes were provoked by adding 5 µl WIS containing 0.5% haemoglobin and the taxic agent to a 495 µl aliquot of the mixture containing promastigotes already adapted to a concentration of the attractant added previously. When experiments required a 5% fall in osmotic pressure, this was obtained by adding 25 µl distilled water to 475 µl of a mixture containing 10^5 cells/ml promastigotes.

3. Results

TSLM measured after stimulus with seven different compounds (Fig. 1) showed that *Le. amazonensis* promastigotes respond to both chemotaxic and osmotaxic stimuli. Low concentrations (0.001 mM) of sucrose, lactose, mannitol, and glycine induced chemotaxic responses although at these levels Hepes, NaCl and guanosine were unable to elicit any type of taxic response. Nevertheless, these substances induced an osmotaxic response at high concentrations (50 mM in the case of NaCl, 100 mM for the other substances). Sucrose, lactose, mannitol, glycine or any other solute can also provoke osmotaxic responses at high concentrations. As can be observed in Fig. 1, the two types of taxic response do not synergize when promastigotes are stimulated simultaneously by 0.001 mM sucrose and 50 mM NaCl. The same can be said when the two types of response are triggered at the same time by high concentrations (100 mM) of sucrose, lactose, mannitol, or glycine.

Promastigotes of *L. amazonensis* responded to 0.001 mM sucrose equally at pH 6.8 or 6.0. The protocol described previously was used in these assays, the only difference being the pH of WIS. TSLM values for the controls (±SE) at pH(s) 6.0 and 6.8 were 11.0 ± 0.7 and 9.4 ± 0.5 ($P = 0.09$), while in the presence of sucrose these values were 14.9 ± 0.9 and 16.1 ± 0.8 ($P = 0.29$), respectively.

A crude salivary gland extract of *Lu. longipalpis* at a concentration equivalent to 25 lobes/ml was also able to elicit a taxic response in promastigotes (TSLM-control $\pm SE = 9.6 \pm 0.4$; TSLM-extract salivary $\pm SE = 14.5 \pm 0.6$; $P < 0.05$). In this case, it was not possible to determine whether the response was chemotaxic or osmotaxic.

The taxic responses of *Le. amazonensis* promastigotes submitted to different concentrations of sucrose, mannitol, or NaCl are shown in Fig. 2. The affinity constant (K_d) of the higher affinity receptors for sucrose was calculated after using the Sigma-Plot program to make a hyperbolic adjustment of the response to rising concentrations of this carbohydrate (Fig. 2A). This constant indicates the sucrose concentration at which half of the higher affinity receptors able to bind to this carbohydrate are occupied. The surprising affinity value of 0.6 ± 0.4 pM was obtained. Although the data shown in Fig. 2B do not allow the affinity constant for mannitol to be calculated, an approximate value of 1.5–4 pM could be estimated. Osmotaxic responses in *Leishmania* are much less sensitive than chemotaxic ones. The osmotaxic response to NaCl was only triggered by

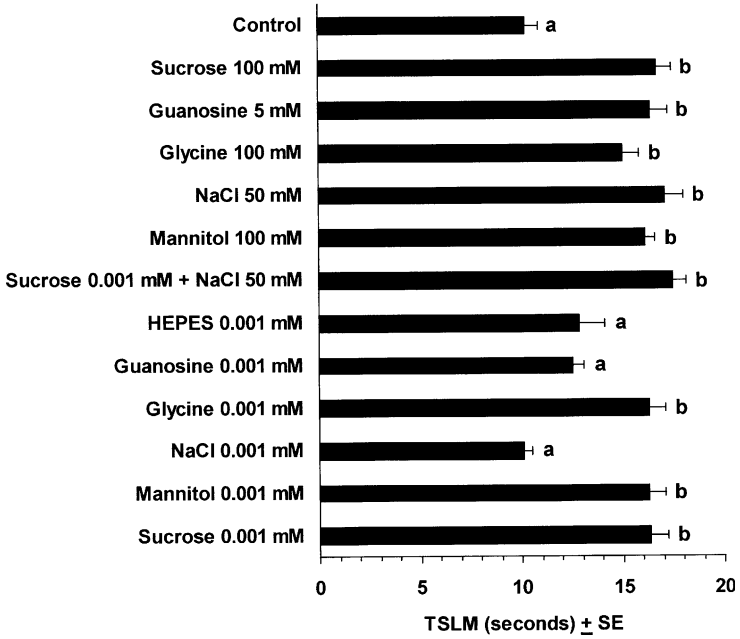

Fig. 1. Mean time of straight line movement (TSLM) of promastigotes of *L. amazonensis* in response to different concentrations of some chemical agents. Promastigotes were washed and incubated in the absence (control) and presence of diverse chemical compounds. These mixtures, containing promastigotes, were used to prepare slides that were observed under the optical microscope (400×) to determine the TSLM under each of the conditions studied. In all 100–250 counts of the TSLM obtained in two independent experiments were performed for each analysis. Identical letters represent identical means, different letters represent means that were significantly different ($P < 0.05$). The data were analyzed using ANOVA and Student's *t* test. SE: standard error.

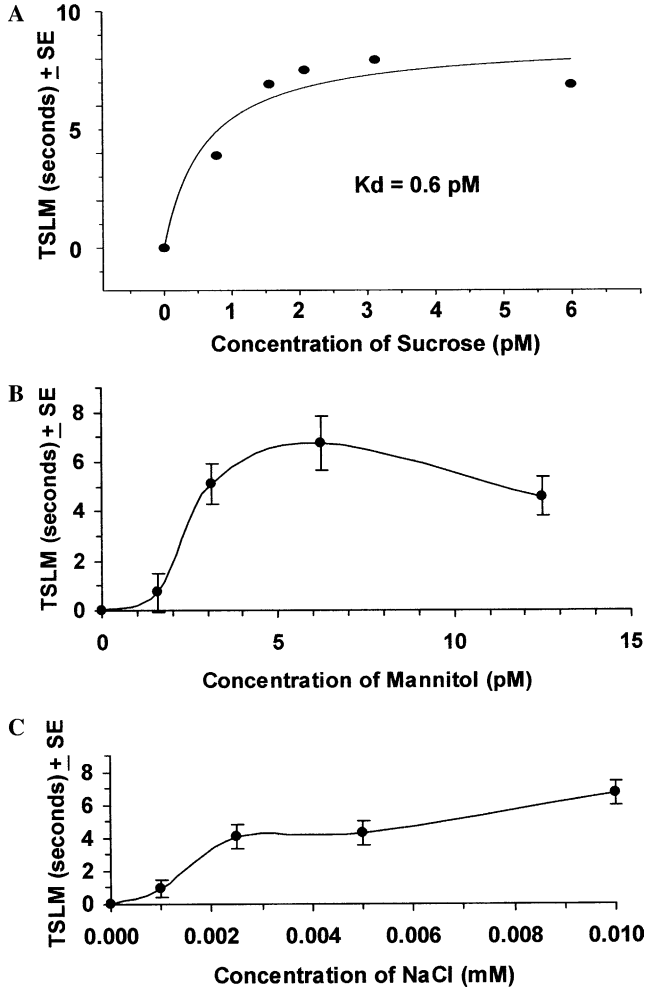

Fig. 2. Mean time of straight line movement (TSLM) of *L. amazonensis* promastigotes in different concentrations of sucrose, mannitol and NaCl. Promastigotes were washed and incubated in the absence (control) or presence of different concentrations of sucrose, mannitol and NaCl. These mixtures were used to prepare slides that were observed under the optical microscope (400×) to determine TSLM values. (B and C) The data presented are results of two independent experiments, representing at least 100 TSLM counts for each point (except for Fig. 2A, which represents the results of three experiments). The TSLM measured for control was subtracted from the other data so that curves commenced at zero. The SIGMA PLOT 8.0 program was used to calculate the affinity constant by fitting the data to a hyperbolic curve. SE: standard error.

concentrations approximately 10^6 times greater than those of sucrose or mannitol used in this experiment (Fig. 2C).

According to the results shown in Fig. 3, promastigotes present adaptation both to chemotaxic and osmotaxic stimuli after remaining at stable concentrations of the stimulant agent for 60 min. The only exception was for 50 mM NaCl (Fig. 3A) that had not presented adaptation after 30 min. It was not possible to measure the TSLM after 60 min in this case, since the promastigotes were losing their mobility.

Promastigotes of *Leishmania* may show a negative osmotaxic response when cells adapted to a certain osmotic pressure are submitted to a slight drop in pressure (Fig. 3C). The fall in TSLM values indicates that they increase the rate of change in direction as if "seeking" for areas where osmotic pressure is greatest. However, this "seeking" phase disappears after 60 min as the promastigotes adapt to this new condition of osmotic pressure.

Further evidence that adaptation is important in promastigote behavior can be observed from the results shown in Fig. 3B. These show that promastigotes adapt to the presence of a certain sucrose concentration and may resume responding to this carbohydrate if its concentration begins to rise again.

4. Discussion

Promastigotes present taxic responses when submitted to environments containing low concentrations of sucrose, lactose, mannitol, and glycine (Fig. 1). However, they were not able to respond to the same molar concentrations of NaCl, Hepes, and guanosine. If the taxic responses to low concentrations of sucrose, lactose, mannitol, and glycine were osmotaxic, as proposed by Leslie et al. (2002), then NaCl, Hepes, and guanosine could also elicit a taxic response under the same experimental conditions. It should be emphasized that, when in solution, NaCl ionizes to Na^+ and Cl^-. This solution has approximately double the osmotic pressure of one with the same molar concentration of sucrose or any other substance that does not ionize. Even so, there was no response to 0.001 mM NaCl. These results present strong evidence that the responses to low (0.001 mM) concentrations of sucrose, lactose, mannitol, and glycine are chemotaxic. This line of argument is even stronger when the data in Fig. 2 are analyzed. The concentrations of sucrose and mannitol (Figs. 2A and B) able to trigger chemotaxic responses are a million (10^6) times lower than those of NaCl that elicit osmotaxic responses (Fig. 2C).

On the other hand, Leslie et al. (2002) were correct in regarding the capacity of promastigotes to respond to osmotaxic stimuli, since higher concentrations of NaCl and guanosine triggered responses that should be considered as osmotaxic (Figs. 1, 2C and 3A).

The behavior of promastigotes when submitted to a 5% decrease in osmotic pressure in the medium to which they were adapted is shown in Fig. 3C. The fall in TSLM relative to the control reveals that promastigotes can be repelled by a negative osmotaxic response.

Information exists in the literature that could explain the mechanism of adaptation to small reductions in osmotic pressure in *Leishmania*. According to Vieira et al. (1996, 1997), promastigotes under hypo-osmotic stress can regulate their cell volumes by liberating aminoacids into the external medium to reduce internal osmolarity. This could also explain why in our experiments a negative osmotaxic response (Fig. 3C) was only observed when we reduced the promastigote concentration from 10^6 to 10^5 cells/ml. The concentration of aminoacids liberated in the experiments with 10^6 cells/ml probably was high enough to interfere with the results (data not shown). Considering that the number of parasites are enormous in sand fly infections, a negative osmotic response may not be usual in vivo.

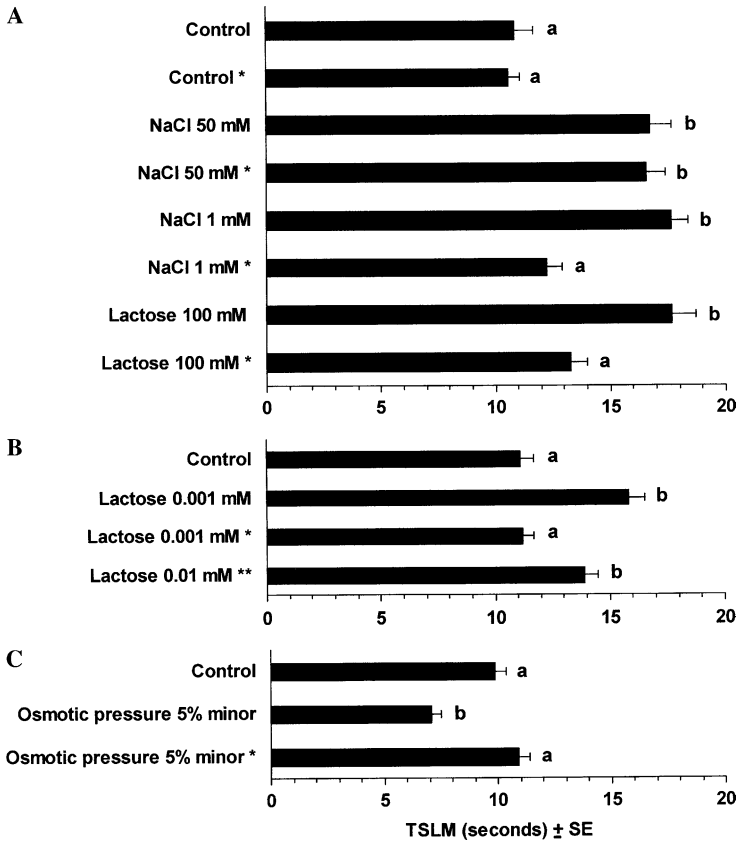

Fig. 3. Adaptation to taxic stimuli in *L. amazonensis* promastigotes The capability to undergo adaptation after different stimuli was investigated in *Le. amazonensis* promastigotes. (A) Promastigotes were not stimulated (control), or stimulated with NaCl or lactose. TSLM was measured immediately after adding the taxic agent and 60 min later (*) (NaCl 50 mM was examined after 30 min). (B) TSLM was measured immediately after adding lactose to a final concentration of 0.001 mM, 60 min after this addition (*) and immediately after further addiction of lactose to this mixture, raising the final concentration from 0.001 mM to 0.01 mM (**). (C) TSLM was measured immediately after a 5% drop in osmotic pressure (provoked by adding water) and 60 min later (*). The data presented are results of two to three independent experiments. Identical letters represent equal means, different letters represent those that are significantly different ($P < 0.05$). Data were analysed using ANOVA and Student's t test. SE: standard error.

Sucrose and lactose were chosen to study the taxic responses in *Leishmania* because they are disaccharides. These sugars cannot be transported to the interior of the promastigotes if they have not previously been digested to monosaccharides (Gontijo et al., 1996). They are thus metabolically inert until hydrolyzed. The fact that these carbohydrates remain outside the cells is an important argument in favor of the presence of membrane receptors on promastigotes, as proposed by Oliveira et al. (2000). Even so, in experiments involving incubation for 60 min, sucrose was substituted by lactose; since promastigotes may produce and excrete a sucrase able to digest sucrose (Gontijo et al., 1996).

The presence of receptors able to interact with the extracellular medium is an important requirement for chemotaxic phenomena (Adler, 1978). Binding of molecules to these receptors would be responsible for triggering these responses. On the other hand, it makes no sense for osmotaxic responses to be elicited by stimulation of receptors, since any osmotaxically active agent, being soluble, could be responsible for this type of response.

According to the results presented in the Fig. 1, when promastigotes were stimulated at the same time by chemotaxic as well as by osmotaxic stimulus, the responses obtained were not accumulative. Although chemotaxic responses are triggered by stimulation of receptors and osmotaxic responses by an unknown mechanism, they appear to converge to a common pathway and consequently do not synergize.

The presence of carbohydrate receptors on the promastigote surface was studied in *Le. donovani* by Schottelius and Gabius (1992). These authors conjugated several different carbohydrates to molecules of the enzyme β-galactosidase and used the activity of this enzyme to calculate the affinity of these molecular hybrids for the promastigote surface. The affinity constants measured in this study varied from 62.5 to 155 nM, values considerably greater than the 0.6 pM obtained in our study for sucrose (Fig. 2A). This great difference could be explained by the presence of the β-galactosidase molecule in the carbohydrate–enzyme conjugate. The enzyme molecule could be provoking an esteric hindrance. Despite this, several molecules of the carbohydrate–enzyme conjugate adhered to promastigotes at 4 pM (Schottelius and Gabius, 1992).

It is currently unknown how many different receptors involved with chemotaxic responses are present on the

promastigote surface. The wide variety of molecules that can elicit responses in promastigotes (Leslie et al., 2002; Oliveira et al., 2000) suggests that they possess low specificity and a wide range of affinities that vary from high to low. The profile proposed above does not require necessarily an existence of a large number of different receptors, since low specificity makes the same receptor able to bind to a large number of different but structurally related molecules, such as the carbohydrates which are all polyhydroxylated molecules. Great differences in affinity would guarantee that, along an ascending gradient of the attractant, there would "always" be receptors not yet saturated by the attractant and thus, not yet "silenced" by adaptation. According this model, the number of receptors "occupied" by attractant molecules along a gradient would go growing step by step.

Aminoacids and carbohydrates probably have independent systems of perception due to their great structural divergence. The presence of several hydroxyl groups on its molecule probably means that mannitol binds to the same complex of receptors responsible for carbohydrate perception.

As well as carbohydrates and aminoacids, the saliva of phlebotomines could play a role in the development of *Leishmania* in the vector, with salivary gland extracts acting as taxic agents. Although concentration of the extract was 25 lobes/ml (each slide prepared with 10 µl had only the equivalent of 0.25 glandular lobes), it is difficult to determine the type of taxic response it elicited, since its osmolarity and composition is unknown.

In nature, *Leishmania* promastigotes live exclusively inside the phlebotomine gut. However, the capacity to respond to gradients of taxic agents may be a characteristic inherited from free-living ancestors. This characteristic probably was maintained in *Leishmania* because it would be an efficient manner for the protozoan to perceive and reach the anterior regions of the vector gut from where they may be transmitted to the vertebrate host.

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