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RESEARCH BRIEF

A Sensitive Method for Assaying Chemotaxic Responses of *Leishmania* Promastigotes

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Oliveira, J. S., Melo, M. N., and Gontijo, N. E. 2000. A sensitive method for assaying chemotaxic responses of *Leishmania* promastigotes. *Experimental Parasitology* **96,** 187–189. This article describes a sensitive, cheap, and easy method for assaying chemotaxic responses of *Leishmania* promastigotes. A gradient of the substance to be assayed was produced inside a series of commercially available capillary tubes submerged in a promastigote suspension. After an incubation period, the attractiveness of the substance under test was measured by counting the number of parasites in the capillaries in a Neubauer chamber. Different responses were detected in two strains of *Leishmania amazonensis* and one strain of *L. chagasi* after standardization of the method to assay attraction to carbohydrates. Very different responses were obtained when the test was performed using promastigotes of the same strain in two different physiological states (log and stationary phase). The stationary phase cells showed an enhanced chemotaxic capability, which can be explained by the fact that the metacyclic forms commonest in stationary phase cultures have greater mobility than other promastigotes. This method will permit studies to be made of the attractive response to different substances in *Leishmania* species and other trypanosomatids and facilitate characterization of the potential receptors involved in the chemotaxic response. An adaptation of the method to assay the response to repellent substances is also provided.

Index Descriptors and Abbreviations: Leishmania amazonensis; Leishmania chagasi; Leishmania development; metacyclic promastigotes; trypanosomatids; carbohydrates; chemotaxic response; chemotaxis; chemoattractant; WIS, washing and incubation solution; CC, chemotaxic capability.

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During the final phase of their development in phlebotomine sand flies, the promastigote forms of *Leishmania* migrate to the anterior regions of the digestive tube, from where they are transmitted to the mammal host during blood-feeding (Killick-Kendrick 1990). This migration is a necessary prerequisite for successful transmission. What stimulates the promastigotes to migrate is not fully understood, although some time ago the hypothesis that they could be attracted to the carbohydrates ingested by phlebotomine sand flies as an energy source stored in the crop diverticulum was raised (Killick-Kendrick 1978). These carbohydrates are liberated into the sand fly mid-gut to be digested and absorbed as required. According to this hypothesis, the liberation of carbohydrates is continuous, creating a gradient in the interior of the digestive tube that promotes migration of the promastigotes to the area where the crop diverticulum opens into the main gut.

R. S. Bray (1983) was the first person to study chemotaxic responses of *Leishmania* promastigotes using a methodology similar to that described by Wilkinson (1974). In this method a promastigote suspension is introduced into a chamber made of a sawn-off disposable 1-ml tuberculin syringe covered at the bottom with a 1.2-μm-pore-size Millipore filter. This apparatus is then immersed up to the level of the promastigote suspension in a solution of the putative chemotaxic fluid. Although it was possible to detect chemotaxic responses by this method, it proved to be somewhat insensitive, requiring high concentrations of attractant ($\geq$20 mM). Even at high concentrations, this method was ineffective in demonstrating the chemotaxic effect exercised by the carbohydrates considered to be important in the diet of phlebotomines, such as glucose and fructose (Bray 1983). Thus, a more sensitive and adequate methodology is required to study chemotaxy of *Leishmania* in detail. The new method proposed here is based on that devised by Adler (1973) to assay chemotaxy in bacteria and later modified by Palleroni (1976) and Mazumder *et al.* (1999). This method is sufficiently sensitive to distinguish clearly (usually with 99% confidence)

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the chemotaxic effect exercised by seven carbohydrates, including glucose and fructose. It uses readily available glass capillary tubes (length 75 mm and diameter 1 mm) normally used in microanalyses of hematocrit values. A typical assay requires 18 capillary tubes, consisting of 6 control tubes interspersed among 12 capillary tubes containing the two substances to be tested (i.e., 6 for each of the substances). Results did not improve significantly when more than 6 capillary tubes per test substance were used (data not shown). Control tubes were prepared by filling them by capillary action with a washing and incubation solution (WIS) containing 30 mM sodium β-glycerophosphate, 87 mM NaCl, 27 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ (pH 7.1), 0.004% enriched bovine serum albumin, and 0.2% agarose. The remaining 12 tubes were filled with this same solution, 6 tubes also containing one carbohydrate to be tested at a concentration of 100 mM and the remaining 6 containing the other carbohydrate at the same concentration. Before the agarose gelled, the solution was carefully pushed to the other extremity of the capillary tubes using atoxic modeling clay (Faber Castell), to an exact distance of 1 cm from the tip (Figs. 1a–1c). The tip of each tube was then sealed with modeling clay and isolated with a strip of Parafilm (Fig. 1d). To form a carbohydrate gradient in the tubes, the open extremities of the 18 capillary tubes were filled with WIS without agarose and wrapped in two supporting strips of corrugated plastic (Fig. 1e) in a 110-mm-diameter petri dish containing 50 ml of the same solution. The apparatus was maintained in position on the bottom of the petri dish by placing a small glass weight on the top

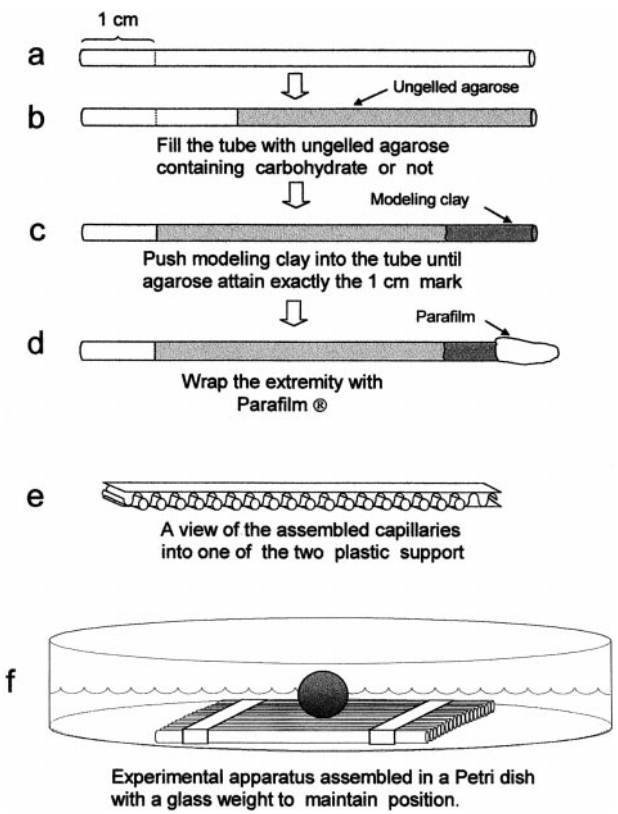

FIG. 1. Experimental apparatus for measurement of chemotaxic responses of *Leishmania* promastigotes.

(Fig. 1f). After 30 min, at 25°C, a carbohydrate gradient had formed in the open extremity of each tube, except in the controls. In the mode for investigating the gradient formation in the capillaries, when glucose was tested, its concentration was measured in the solution present in the open extremity of the tube (the colorimetric kit from Sigma Cat. No. 315-100 was adequate). After 30 min, the concentration observed varied from 3.7 $\pm$ 0.4 mM in the first 0.5 cm of the open extremity of the tube to 16 $\pm$ 1.1 mM in the 0.5 cm closest to the agarose gel. So, the glucose concentration in the tip of the capillary was considerably less than 3.7 mM. This gradient remained reasonably stable during the entire experiment. After formation of the gradient, the apparatus containing the capillary tubes and their supports was removed from the petri dish and a suspension of *Leishmania* promastigotes, previously washed with WIS, was added to the dish to a final concentration of 5×10^5 cells ml^{-1}. These cells were cultivated in MD medium enriched with 11 mM sucrose and 5% foetal calf serum (Melo *et al.* 1985). Care was taken not to homogenize the suspension with circular movements since this would have caused the cells to accumulate in the center of the dish. After homogenization the capillary tubes and supports were immersed in the promastigote suspension for an incubation period of 1 h. Longer incubation periods produced less clear results (data not shown). After incubation, the capillary tubes and supports were carefully removed from the dish and the open extremities of the capillary tubes were inverted and maintained in the vertical position until their contents (approximately 7.8 μl) could be removed individually with a fine-tipped automatic pipette (electrophoresis gel-loading pipette and tips, Cat. Sigma code P2543 and T3778, respectively) and transferred to 0.5-ml microcentrifuge tubes containing 31.2 μl of solution (36 mM citrate buffer, 120 mM NaCl, and 0.2% formaldehyde, pH 7.2) for counting. The cells attracted into each capillary tube were counted twice in a Neubauer chamber. In all the experiments the tubes containing the carbohydrates contained significantly more promastigotes than the controls (usually $P \leq 0.01$, rarely $P \leq 0.05$). Each experiment was carried out in duplicate on different days and the means obtained were compared by Student's t test. To compare experiments carried out on different days, the chemotaxic action of the carbohydrates tested was expressed as chemotaxic capability (CC), this representing the number of promastigotes attracted to each test capillary tube divided by the number present in the respective control tube.

Two strains of *Leishmania amazonensis* (WHO reference strains IFLA/BR/67/PH8 and MHOM/BR/1960/BH6) and one strain of *L. chagasi* (MHOM/BR/1970/BH46) were used to test the method here described using different carbohydrates as chemotaxic agents. As can be observed from Table I, the method was highly sensitive in detecting chemotaxic activity. This activity was significantly greater in relation to the controls for all the carbohydrates tested and for each of the strains used. It is interesting to note that, although the di- and trisaccharides are not absorbed by the promastigote forms before being digested to monosaccharides (Gontijo *et al,* 1996), they generated a very intense chemotaxic effect (Table I). This effect probably acts through the activation of receptors located on the surface of the promastigote cell membrane. A chemotactic system like this was already described for bacteria (Hazelbauer and Harayama 1983). Since the cells respond with different intensities to the stimuli generated by different carbohydrates, the affinity of the receptors for the carbohydrates may be variable or there may be more than one type of receptor.

Curiously, there was a greater difference between the responses obtained for promastigotes of the same strain of *L. amazonensis* in different physiological states (logarithmic phase and stationary growth

TABLE 1
Chemotaxic Capability of Some Carbohydrates on Promastigotes of *Leishmania amazonensis* and *L. chagasi*

Carbohydrate	Chemotaxic capability (CC) ± SE							
	Glucose	Fructose	Sucrose	Raffinose	Manose	Galactose	Maltose	Melibiose
L amazonensis PH8 (log phase)	2.4 ± 0.1	2.4 ± 0.1	4.2 ± 2.1	3.0 ± 1.1	—	—	—	—
L amazonensis PH8 (stationary phase)	7.5 ± 1.4	7.7 ± 0.8	5.2 ± 2.0	4.4 ± 2.1	—	—	—	—
L amazonensis BH6 (log phase)	7.1 ± 0.2	5.6 ± 0.7	7.5 ± 0.1	6.8 ± 1.0	2.8 ± 0.7	4.0 ± 1.2	7.0 ± 1.2	3.9 ± 0.3
L chagasi BH46 (log phase)	3.1 ± 1.4	3.1 ± 1.3	2.7 ± 0.5	2.8 ± 0.5	2.7 ± 1.1	2.4 ± 0.6	3.1 ± 0.9	4.2 ± 1.2

Note. The CC represents how many times the number of attracted promastigote cells was greater than the number present in the respective control.

phase) than between the two *Leishmania* species in the same physiological state (Table I). This may be a reflection of the greater mobility of the metacyclic forms that are more numerous in stationary growth phase cultures (Killick-Kendrick and Rioux 1991). An adaptation of this methodology to assay repellent substances could be easily accomplished. An approach would be to compare the number of promastigotes within the control tubes with the number within the tubes containing the putative repellent. Another possible approach for this would be to supply a known number of promastigotes to the vacant interior of the tubes. After an incubation time, the number remaining within the tubes should be compared with the number within the control tubes.

This new method will allow comparative studies to be done with different species of *Leishmania* and probably with other trypanosomatids. Substances other than carbohydrates that could be tested include the saliva of phlebotomines, which may also contain chemoattractant substances. The method will also permit the characterization of the cell surface receptors involved in chemotaxic responses.

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