*Mem. Inst. Oswaldo Cruz*, Rio de Janeiro, Vol. *88* (3): 407-412, jul./set. 1993

# IMMUNOGOLD LABELING AND CERIUM CYTOCHEMISTRY OF THE ENZYME ECTO-5'-NUCLEOTIDASE IN PROMASTIGOTE FORMS OF *LEISHMANIA* SPECIES

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*We have applied both enzyme cytochemistry and immunological labeling techniques to characterize the enzyme 5'-nucleotidase (5'-Nase), at the ultrastructural level, in promastigote forms of four* Leishmania *species:* Leishmania amazonensis, Leishmania mexicana, Leishmania donovani *and* Leishmania chagasi. *The cerium phosphate staining was localized at the surface of the cell body, the flagellum and the flagellar pocket membranes of all the parasites studied. The immunogold labelling technique confirmed these results. In this report we localized 5'-Nase in* L. chagasi *and* L. amazonensis *which have been implicated respectively in visceral and cutaneous forms of leishmaniasis. In addition, we confirmed the localization of this phosphomonoesterase in the other two species studied. The superior quality of the images, obtained with both methodologies, confirms that these parasites possess mechanisms capable of hydrolyzing nucleotide monophosphates, and that the expression of 5'-Nase is associated with the outer surface of the plasma membrane.*

Key words: *Leishmania* – 5'-nucleotidase enzyme – ultrastructural cytochemistry – immunogold labeling

A variety of enzymes involved in purine metabolism has been identified in promastigotes of various *Leishmania* species (Marr et al., 1978). A nucleotidase activity from *L. tropica* promastigotes was reported by Pereira & Konigk (1981) and distinct 5'- and 3'-nucleotidase (5'-Nase and 3'-Nase) activities, were identified at the surface of *L. donovani* promastigotes (Gottlieb & Dwyer, 1983; Dwyer & Gottlieb, 1984).

The 3'-Nase, recently purified to SDS/PAGE homogeneity from *L. donovani,* seems to be relevant in the diagnosis of human visceral leishmaniasis by allowing the detection of the amastigote stage in the host tissues that apparently have little or no such enzyme activity themselves (Gottlieb, 1985; Gbenle & Dwyer, 1992).

The ectoenzyme 5'-Nase (5' ribonucleotide phosphohydrolase EC 3.1.3.5) was found in a wide variety of mammalian cells (Widnell, 1972; Gurd & Evans, 1974; Stanley et al., 1983; Gandarias et al., 1985; Cramer et al., 1988; Le Hir & Kaissling, 1989), and has been used as a plasma membrane enzyme marker in biochemical and morphological studies, and also for the characterization of the degree or state of cell maturation (De Pierre & Karnovsky, 1973; Gottlieb & Dwyer, 1983; Côrte-Real et al., 1988). An intracellular pool of this membrane-bound enzyme has also been detected in several cell types (Edelson & Cohn, 1976; Meirelles & De Souza, 1986; Stanley et al., 1980; Widnell, 1982).

*Leishmania* species require exogenous purine for their growth *in vitro* (Steiger & Steiger, 1977) and the 5'-Nase hydrolyzes monophosphate 5'-ribonucleotides to nucleosides making the latter available for the parasite. In fact, some studies have indicated that amastigotes and promastigotes of *L. mexicana* exhibit both enzymatic activities and extracts of *L. m. amazonensis* showed 5'-Nase activity (Hassan & Coombs, 1987). In the present paper we set

This research was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP) and Fundação Oswaldo Cruz (FIOCRUZ).
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Received 28 December 1992.
Accepted 15 February 1993.

out to localize through ultrastructural cytochemistry the 5'-Nase in four species of Leishmania. Parasites were identified by species specific monoclonal antibodies and by zymodeme and schizodeme analysis (Grimaldi et al. 1987, 1991): *L. amazonensis* and *L. mexicana* (implicated in cutaneous and/or mucocutaneous forms of the disease) and *L. donovani* and *L. chagasi* (associated with visceral leishmaniasis).

In addition, since there is no cytochemical report on the presence of 5'-Nase in *L. amazonensis* and *L. chagasi,* we used cerium choride to detect the enzymatic activity. We also worked with an immunological labeling technique, using a rabbit antiserum against 5'-Nase and Ptn-A colloidal gold as labelling agent.

## MATERIALS AND METHODS

*Parasites* – The *L. amazonensis* (IFLA/BR/67/PH8) and (MHOM/BR/77/LTB0016), *L. chagasi* (MHOM/BR/74/PP75), *L. donovani* (MHOM/IN/80/DD8) and *L. mexicana* (MNYC/BZ/62/M379) used in all experiments was suppplied by Dr Gabriel Grimaldi (Dept. of Immunology, Fundação Oswaldo Cruz, RJ, Brazil). Promastigotes were obtained on the 7th day of cultivation in NNN blood agar medium with an overlay of modified LIT liquid medium (Jaffe et al., 1984).

*Enzyme cytochemistry* – Parasites were incubated for detection of 5'-Nase activity in the modified medium described by Uusitalo & Karnovsky (1977) at 37 °C for 60 min. The medium has the following composition: 100 mM tris-maleate buffer (pH 7.2), 5 mM adenosine 5'- monophosphoric acid (AMP) as substrate, 10 mM Mg(NO3)2 and 1mM CeCl3. The following controls were used: (1) incubation in substrate-free medium; (2) incubation in medium containing β-glycerophosphate (β-GP) instead of AMP; (3) incubation in the complete AMP or β-GP medium with addition of 0.5 or 2.0 mM levamisole, an inhibitor of nonspecific alkaline phosphatase (Borgers, 1973). All reagents were obtained from Sigma Chem. Co. Samples of mouse splenic tissue were used as positive control.

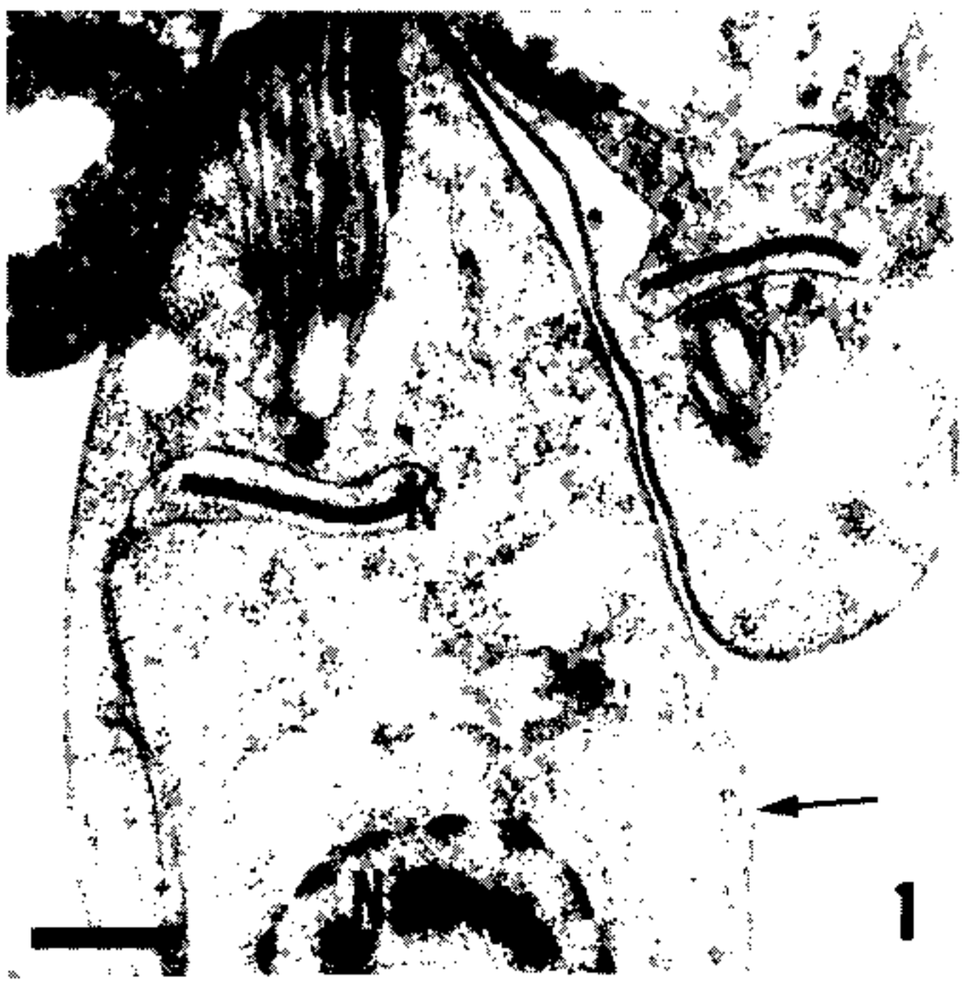
*Electron microscopy* – Parasites were fixed in 1% paraformaldehyde (PFA) and 0.5% glutaraldehyde (GA) in 0.1 M tris- maleate buffer pH 6.0 for 30 min at 4 °C. They were washed with buffer and used for the cytochemical experiments. After incubation of the cells in the media described above, followed by 2.5% glutaraldehyde fixation, the cells were rinsed in cacodylate buffer. The cells were post-fixed with 1% OsO4, dehydrated in acetone, and embedded in Epon. Thin sections were obtained in a Reichert Ultramicrotome OmU3, stained with aqueous uranyl acetate and lead citrate and examined in a EM 10C Zeiss Electron Microscope.

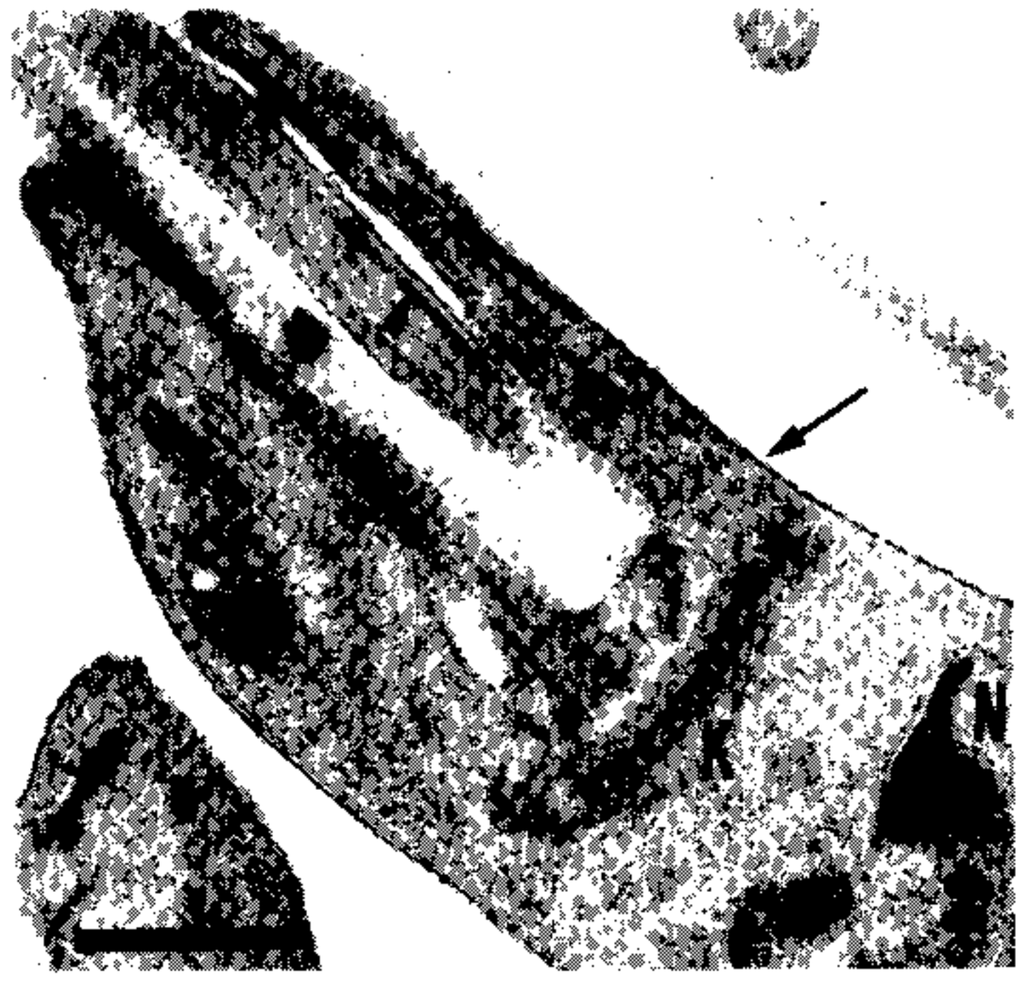
*Immunogold labeling* – The cells were fixed for 15 min in 1% PFA and 0.5% GA in 0.1M sodium cacodylate buffer pH 6.0 for 30 min at 4 °C, dehydrated in methanol, embedded in Lowicryl resin. The labelling was made directly on the grids, the cell sections were incubated first with a rabbit antiserum against rat liver 5'-Nase, kindly provided by Drs Paul Luzio and Keith Stanley (Cambridge, England), using a dilution of 1:10 for 90 min at 20 °C. Protein A complexed to colloidal gold particles was used as the labelling agent at a dilution 1:2, during 30 min. The cell sections were rinsed and then stained with aqueous uranyl acetate before observation.

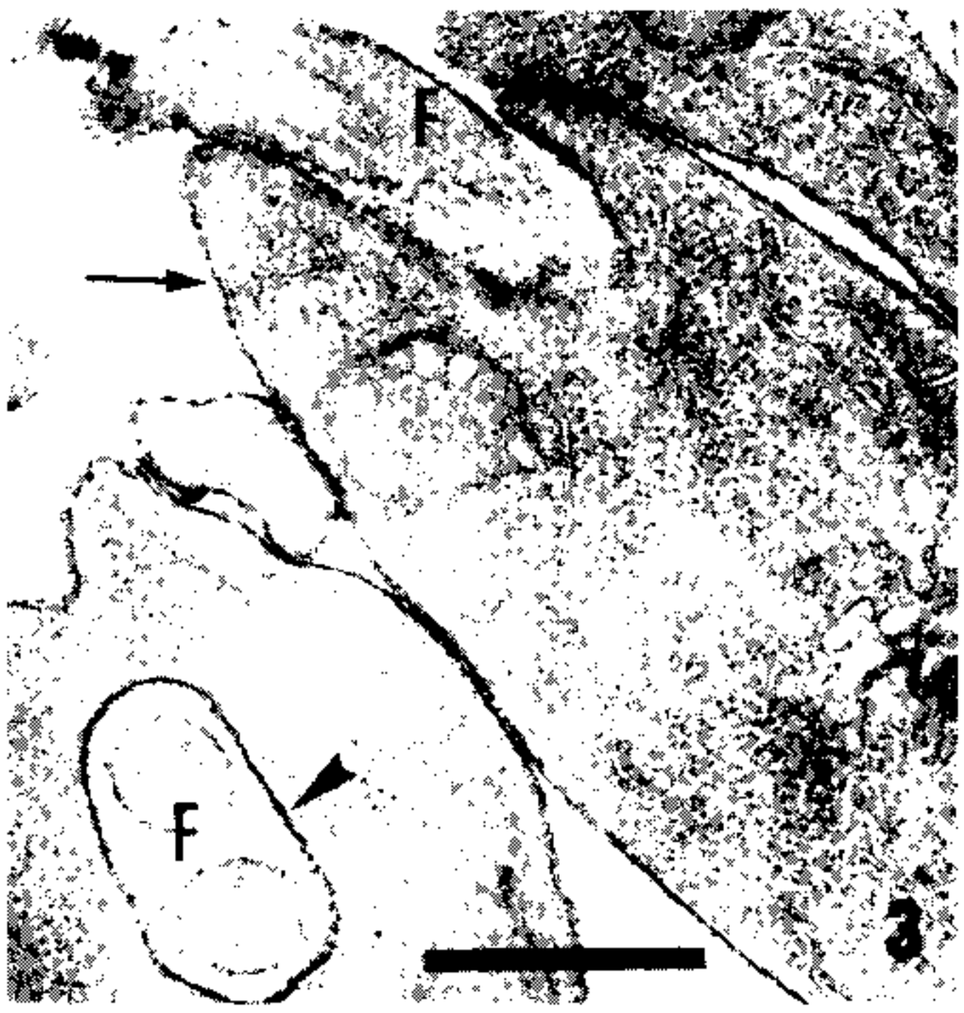
## RESULTS

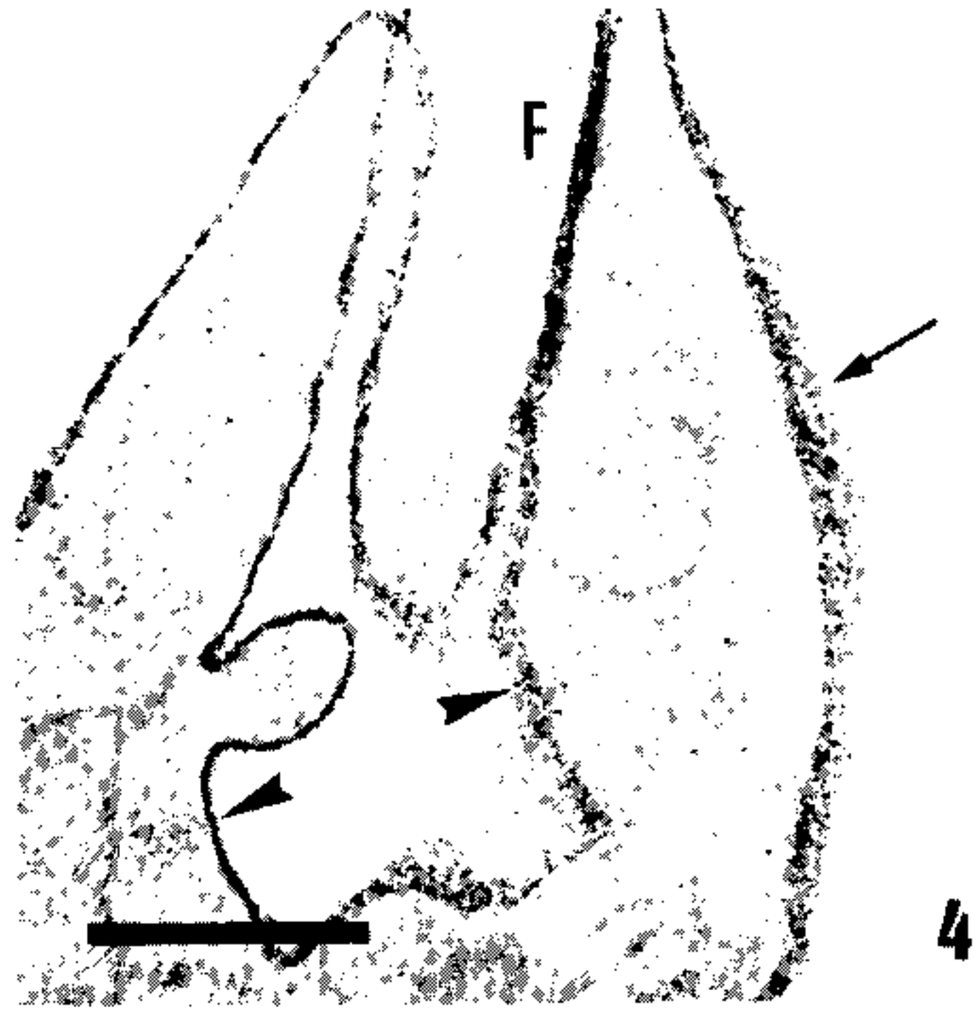
*Enzymatic staining with cerium* – No reaction product indicative of enzyme activity was seen in controls performed in a medium containing β-glycerophosphate instead of AMP, in the complete medium plus levamisole, or in the medium lacking the substrate (Fig. 1). The enzyme was revealed by cerium phosphate deposits observed on the surface of the cell body, the flagellum and the flagellar pocket of *L. chagasi* (Fig. 2), *L. donovani* (Fig. 3), *L. mexicana* (Fig. 4) and *L. amazonensis* (Fig. 5). It seems that there was no quantitative differences in the expression of the 5'-Nase activity in the four species of the parasite. Mouse splenic cells were very reactive for this ectoenzyme (data not shown).

*Immunogold labeling* – The enzyme was localized in *L. amazonensis* (Fig. 6) and *L. mexicana* (Figs 7, 8) Colloidal gold particles were distributed on the external surface of the parasite plasma membrane (Fig. 6), the flagellar pocket membrane and the flagellum (Figs 7, 8). Also, labelling was observed in some vesicles in the parasite's cytoplasm.



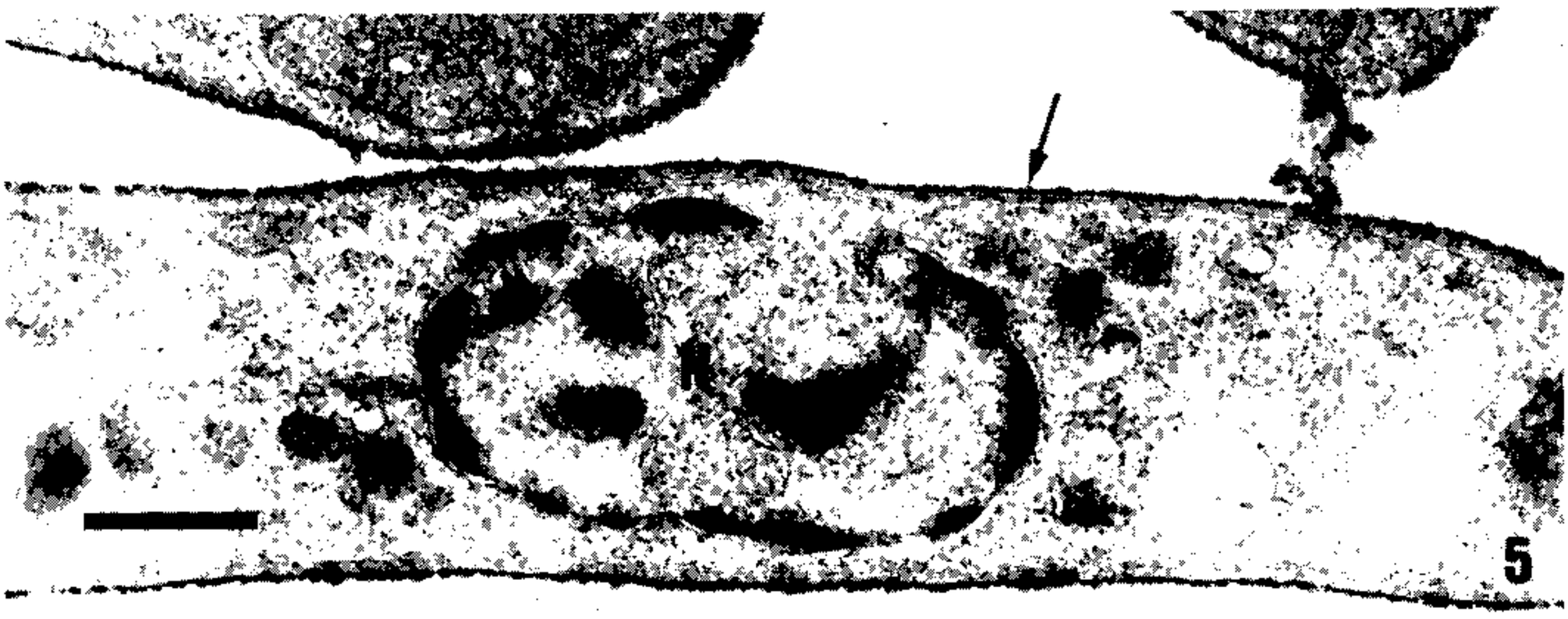




Fig. 1: *Leishmania amazonensis* incubated in a medium lacking nucleotide substrates showed no reaction product on the plasma membrane, flagellar pocket and flagellum membrane. Bar = 0.5 µm. Cytochemical localization of 5'-AMP nucleotidase in promastigotes of four species of *Leishmania* using cerium chloride as the capture agent. Figs 2, 3: Enzyme activity was distributed over the external surface of the plasma membrane of the parasite including the flagellar pocket and flagellar (F) membranes of *L. chagasi* and *L. donovani*. Figs 4, 5: *L. mexicana* and *L. amazonensis* exhibited uniform staining in their plasma membrane, flagellum and flagellar pocket. Bars = 0.5 µm.


*Leishmania amazonensis* (Fig. 6) and *L. mexicana* (Figs 7, 8) showed immunogold labeling on the plasma membrane, flagellar pocket and flagellum. This distribution of labeling is similar to the pattern of the enzyme cytochemical reaction. Bars = 0.1 μm.

## DISCUSSION

In this paper we reported for the first time the presence of 5'-Nase in *L. chagasi* (Cunha & Chagas, 1937) and *L. amazonensis* (Lainson & Shaw, 1972). We confirmed also the previously reported existence of this enzyme in *L. mexicana* and *L. donovani* (Gottlieb & Dwyer, 1983; Hassan & Coombs, 1987). The 5'-Nase has been localized specifically on the parasite surface membrane in all the species studied here. The lead-phosphate reaction, giving an aggregate and clumpy appearance, has been a drawback in previous cytochemical methods (Angermuller & Fahimi, 1984; Cramer et al., 1988). For this reason we used cerium chloride as a capture agent. This reagent also gives a superior quality in the localization of the

enzyme. The results obtained using the enzyme cytochemical method were confirmed by immunogold labeling: the distribution sites of the enzymatic activity were identical in both methods.

In the case of protozoa of the *Leishmania* genus it has been shown that promastigotes require exogenously supplied purines for their continuous *in vitro* growth. Indeed, since *Leishmania* organisms are unable to synthesize the purine ring *de novo* (Steiger & Steiger, 1977; Hammond & Gutteridge, 1984), components of their surface membrane presumably participate in the acquisition of this essential nutrient. An important contribution to cellular purine metabolism is the hydrolysis of extracellular 5'AMP mononucleotide by 5'-Nase (Hassan & Coombs, 1985) which might represent the initiating component of the parasite purine "salvage" pathway that dephosphorylates available nucleotides into nucleosides which could be translocated across the surface membrane (Dwyer & Gottlieb, 1983). This enzyme plays a role in processing exogenously available nucleotides into an adequate form of transport into the cell, thus allowing a source of usable energy to be supplied for the parasite.

In conclusion, the importance of the present study could be considered as relying on two aspects: (a) the report of the existence of 5'-Nase in *L. chagasi* and *L. amazonensis* together with the confirmation of its presence in the two other members of the *Leishmania* genus studied may contribute to the understanding of the mechanisms involved in the parasite's survival in mammalian hosts and (b) the development of reliable methods of detection of these surface ligands as presented here, could help further studies on the role of these molecules, which seem to be necessary for the establishment and maintenance of the infection, raising the possibility of identifying new means of controling infection and disease.

ACKNOWLEDGEMENTS

To Mr Levi Marques Silva, Generval Luciano Batista and Claúdio Belo dos Santos for their excellent technical assistance in these studies, and to Dr H. Momen and Dr Claudio Ribeiro for the review of this manuscript.

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