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Trypanosoma cruzi and American *Leishmania* spp: Immunocytochemical Localization of a Laminin-like Protein in the Plasma Membrane

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BRETAÑA, A., AVILA, J. L., ARIAS-FLORES, M., CONTRERAS, M., AND TAPIA, F. J. 1986. *Trypanosoma cruzi* and American *Leishmania* spp.: Immunocytochemical localization of a laminin-like protein in the plasma membrane. *Experimental Parasitology* 61, 168–175. Patients with Chagas' disease or different clinical forms of American cutaneous leishmaniasis have high antilaminin antibody levels. An immunogold technique employing a specific antilaminin antibody was used in the present study to determine the presence, and define the ultrastructural localization, of laminin-like molecule(s) in American *Leishmania* spp. and *Trypanosoma cruzi*. Laminin was found located specifically in *T. cruzi* trypomastigotes on the external surface of the plasma membrane, close to the sites where the flagellar veil attaches to the plasma membrane. Laminin immunoreactivity was rapidly lost when trypomastigotes were cultured in liquid medium and no reactivity was found in fresh epimastigotes. Promastigotes and amastigotes of American *Leishmania* spp. also showed a specific localization of laminin immunoreactivity, this being limited to the lips of the flagellar pocket and to the parasitic side exactly opposite to the flagellar exit. These results confirm the presence of a laminin-like molecule(s) in both trypanosomatids, the specific localization suggesting a presently unknown function for this protein.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Trypanosoma cruzi;* American *Leishmania* spp.: Laminin; Ultrastructural localization; Immunogold procedures; Trypomastigotes; Epimastigotes; Promastigotes; Amastigotes; Phosphate-buffered saline (PBS).

INTRODUCTION

Laminin, a basement membrane glycoprotein which mediates the attachment of epithelial and endothelial cells to type IV collagen (Terranova *et al.* 1980), was originally identified in cell cultures (Timpl *et al.* 1979) and isolated as a native protein from mouse Engelbreth–Holm–Swarm sarcoma (Chung *et al.* 1979). This protein contains distinct polypeptide chains with molecular weights of about 220,000 (light or B chain) and 440,000 (heavy or A chain), and a carbohydrate moiety comprising 13% by weight of the protein.

Recently, it was demonstrated that sera from humans with acute Chagas' disease and from rhesus monkeys experimentally infected with *Trypanosoma cruzi* contain IgM and IgG antibodies that react with laminin but not with various other purified connective tissue components such as collagen types I, III, IV, and V, fibronectin, heparin sulfate (BM-1) proteoglycan, or chondronectin (Szarfman *et al.* 1982). Similar results have been reported in patients with different clinical forms of American cutaneous leishmaniasis or chronic Chagas' disease (Avila *et al.* 1984) and in patients with *Trypanosoma rangeli* infection (Avila *et al.* 1986), or rhesus monkeys infected with *T. rhodesiense* (Szarfman *et al.* 1982). These results link kinetoplastid infections with the presence of antilaminin antibodies, and, consequently, it was concluded that because the parasite encounters basement

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membranes during its penetration into the host, the production of a laminin-like protein or incorporation of host laminin (or both) may permit the parasite to interact with the host tissue (Szarfman *et al.* 1982). Evidence for the second possibility has recently been obtained by the demonstration that *T. cruzi* trypomastigotes, emerging after intracellular replication in WOS sarcoma monolayers, express a sarcoma associated surface antigen (Chess *et al.* 1983).

By using an electron immunocytochemical method, we present results showing that trypomastigotes (but not epimastigotes) of *T. cruzi*, and amastigotes and promastigotes of American *Leishmania* spp. bear on their plasma membranes a specifically localized protein that reacts with antibodies to laminin.

Materials and Methods

The origins and characteristics of *Trypanosoma cruzi* strains EP, FL, A-35, Y, and Ya, which are representative of zymodemes 1 and 2, respectively, have been described previously (Avila *et al.* 1981b, 1983). Epimastigotes were cultured in Minimum Essential Medium supplemented with 2.5% fetal bovine serum (Avila *et al.* 1979) or in modified liver infusion-tryptose medium (Avila *et al.* 1979) (as was done for *T. rangeli*) and harvested by centrifugation (2400*g* for 20 min 4 C). Trypomastigotes were isolated from infected NMRI-IVIC mice inoculated 10–20 days previously with 0.1×10^6 parasites/mouse. Blood obtained from the axillar plexus was centrifuged on Ficoll–Hypaque discontinuous gradients (Hungerer *et al.* 1981) or on Percoll gradients (Fish *et al.* 1982). Trypomastigotes were also obtained from infected Vero cell cultures, always after more than 20 passages, as described previously (Piras *et al.* 1982).

The American *Leishmania* spp. (EB, AZV, MP, and MA strains of *L. mexicana* and NR strain of *L. braziliensis*) promastigotes were grown at 28 C (Avila and Casanova 1982). Amastigotes were obtained from golden hamsters inoculated in the dorsum of each hind paw with 1×10^5 parasites. After 1–3 months of infection, established lesions were excised, homogenized, and amastigotes purified on Percoll gradients as described previously (Coello and Urbina 1982).

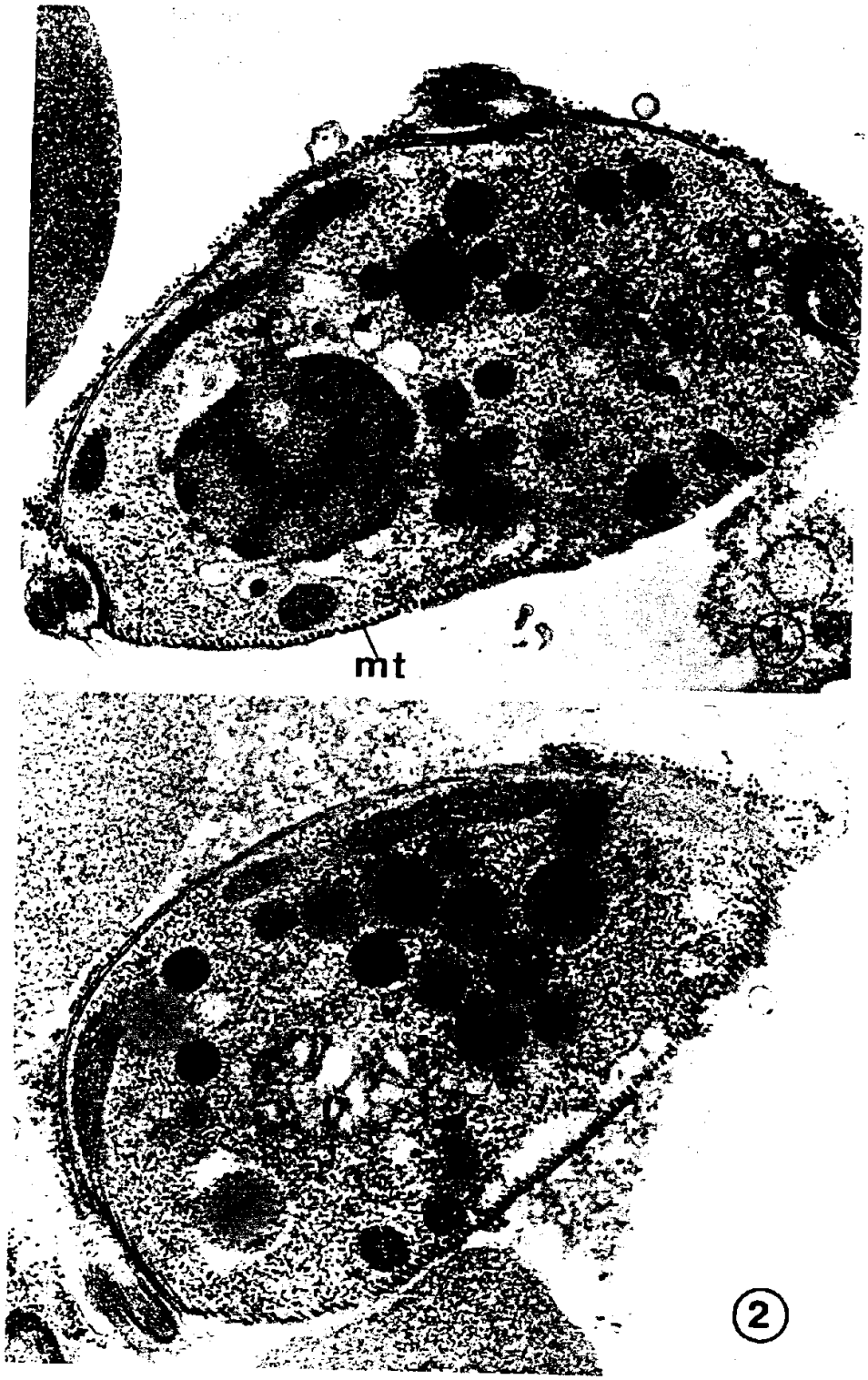
Antibodies to mouse laminin were raised in rabbits, using as antigen laminin purified from a murine Engelbreth–Holm–Swarm tumor (Avila *et al.* 1984). The specificity of the antiserum was verified using Ouchterlony immunodiffusion with purified antibody to laminin (100 μg/ml) which was tested against laminin, fibronectin, and type IV collagen. Using this technique, only a single precipitation line was observed between laminin and antilaminin. As a further control of specificity, antilaminin antiserum (100 μg/ml protein), incubated with purified laminin (500 μg/ml), reduced the specific immunoreactivity measured by the enzyme linked immunosorbent assay by more than 95%. Using immunoelectrophoresis, a single precipitation line was observed using laminin in the well and antibody to laminin in the trough.

In the immunogold staining procedure for electron microscopy, cells were used either unfixed or fixed in 2.5% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.4, for 30 min at room temperature.

The parasites were carefully washed with fresh 0.01 *M* sodium phosphate-buffered 0.14 *M* sodium chloride, pH 7.4 (PBS), incubated for 1 hr with 50 μl of antilaminin (1:20 dilution of stock antisera containing 65 mg/ml of protein), then again washed three times with PBS. Cell pellets were then exposed to 50 μl of 1:2 dilution, in PBS, of gold-coupled goat anti-rabbit immunoglobulin (GAR G 20, 20 nm average, donated by Dr. Jan De Mey, Janssen Pharmaceutica, Beerse, Belgium) for 1 hr. Subsequently, the cells were washed three times in PBS, fixed with freshly prepared 2.5% (V/V) glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2, for 30 min at room temperature, postfixed with 1% OsO_4 in cacodylate buffer, pH 7.2, washed, and then included in 1.5% agar, dehydrated, and embedded in LX-112 resin. The samples were cut in sections of 40–90 nm and stained with uranyl acetate and lead citrate. Electron micrographs were taken with a Hitachi H-500 transmission electron microscope at 75 KV.

Results

When *Trypanosoma cruzi* trypomastigotes isolated from blood of infected mice were examined for the presence of laminin-like protein, a strong positive reaction was found restricted to determined areas on the external surface of the plasma membrane (Figs. 1 and 2). A possible explanation for the presence of laminin-like protein in *T. cruzi* trypomastigotes is that the parasite incorporates host protein. Experiments were performed using Vero cell derived trypomastigotes which had been maintained in cell culture for at least 160 days. These trypomastigotes had, therefore, not been in contact with basement membrane laminin. In these trypomastigotes, it was found that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laminin-like protein immunoreactivity was present in a diffuse form all over the parasitic surface (Fig. 3), suggesting a wider distribution of laminin-like proteins in these organisms, and also that laminin-like proteins are indeed of parasitic origin. It is relevant to note, however, that in contrast to blood derived trypomastigotes, all of which bear antilaminin-reactive proteins, only a low percentage of Vero cell derived trypomastigotes were anti-laminin positive.

The blood-derived trypomastigotes presented laminin-like protein immunoreactivity independently of the zymodeme type to which they belong. In contrast, epimastigotes of all *T. cruzi* strains studied lacked any laminin immunostaining, suggesting that the laminin-like proteins are present or reactive exclusively on the trypomastigote stage. This was corroborated when it was observed that laminin immunoreactivity totally disappeared in blood trypomastigotes cultured in modified liver infusion–tryptose medium for a period as short as 2 days, when trypomastigote/epimastigote transformation was in progress, thus providing indirect evidence of rapid changes in plasma membrane protein components during trypomastigote–epimastigote transformation.

When *T. rangeli* culture forms were examined for anti-laminin antibodies, no reaction was found in all parasites studies.

When amastigote and promastigote stages of *Leishmania braziliensis* and *L. mexicana* were studied, laminin immunoreactivity was found in both, this being again limited to certain areas of the plasma membrane. These correspond to the external lips of the flagellar pocket in promastigotes (Fig. 4) and amastigotes. Also, localization to particular plasma membrane areas, located on the side opposite to the flagellum, was observed (Figs. 5 and 6).

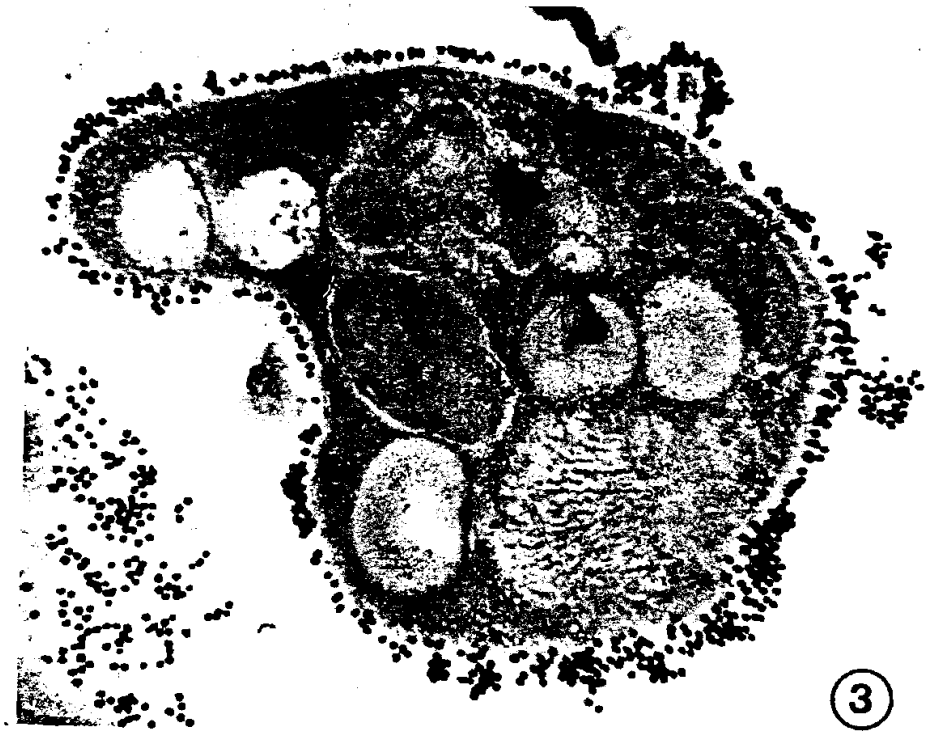
This anti-laminin reactivity was found in all the strains of American *Leishmania* spp. studied.

Discussion

The presence of laminin-like protein(s) in different members of the family Trypanosomatidae is reported. This immunocytochemical study demonstrates that laminin-like protein(s) are located in certain specific areas of the plasma membrane, and, in the genus *Trypanosoma*, this protein is stage-specific, as it is easily demonstrated in blood derived trypomastigotes but not in culture forms of *T. cruzi* and *T. rangeli*. Szarfman *et al.* (1982) have demonstrated that both IgM and IgG antibodies to laminin from *T. cruzi* infected monkeys, purified by laminin-affinity chromatography, produced very strong reactions with trypomastigotes, but showed only weak reactions with epimastigotes when used in the indirect immunofluorescence technique.

The actual localization of laminin-like protein(s) at the external surface of the plasma membrane is, however, distinct in the different members of the family Trypanosomatidae. It is located in both lips of

FIGS. 1–6. Pre-embedding immunogold staining for laminin. FIGS. 1 and 2 *Trypanosoma cruzi* blood trypomastigotes. Note that only the plasma membrane's external surface reacted with antibodies to laminin. No intracellular staining was seen. mt, microtubules. Fig. 1: ×22,500 and Fig. 2: ×25,500. FIG. 3. *Trypanosoma cruzi* trypomastigote from culture. The diffuse positive reaction with antibodies to laminin is seen surrounding the whole parasitic body, including the flagellar membrane, f. ×57,800. FIG. 4. *Leishmania mexicana* promastigote. Observe that the positive antilaminin reaction is specifically located on both external lips (arrows) of the flagellar pocket, fp. No intracellular staining is seen. ×18,000. FIG. 5. *Leishmania braziliensis* amastigote. Note that, due to the sectioning plane, the positive antilaminin reaction is located on one pole of the parasite. ×36,000. FIG. 6. *Leishmania mexicana* amastigote. The positive antilaminin reaction is localized specifically on two opposite poles of the parasite, one near the flagellar pocket, fp (as in promastigotes) and the other on the exact opposite side (arrows). Nucleus, N. ×40,000.



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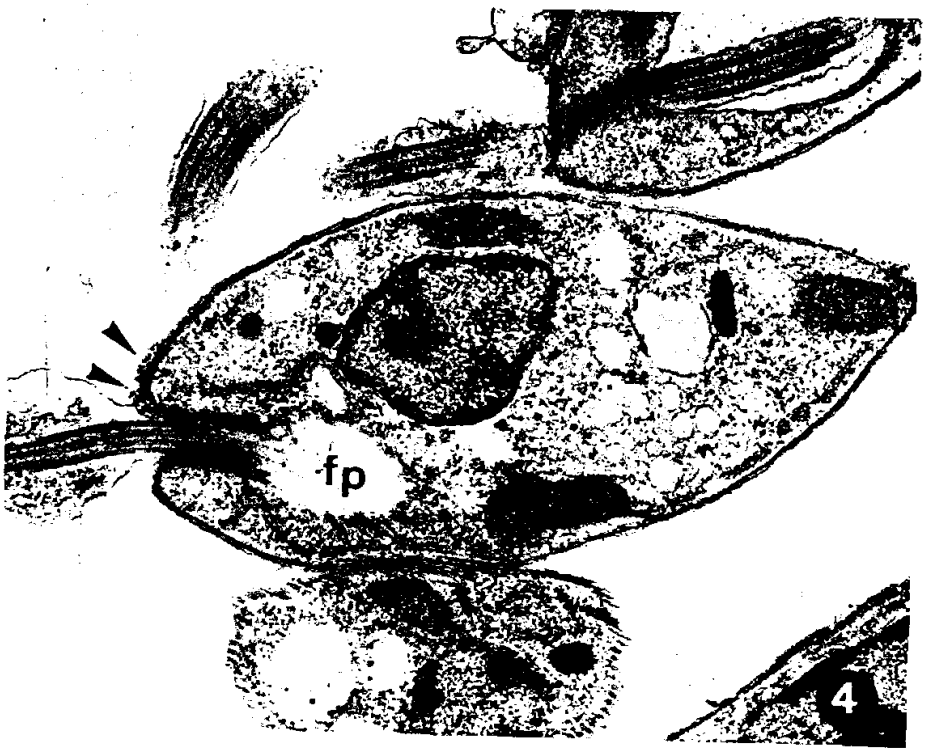

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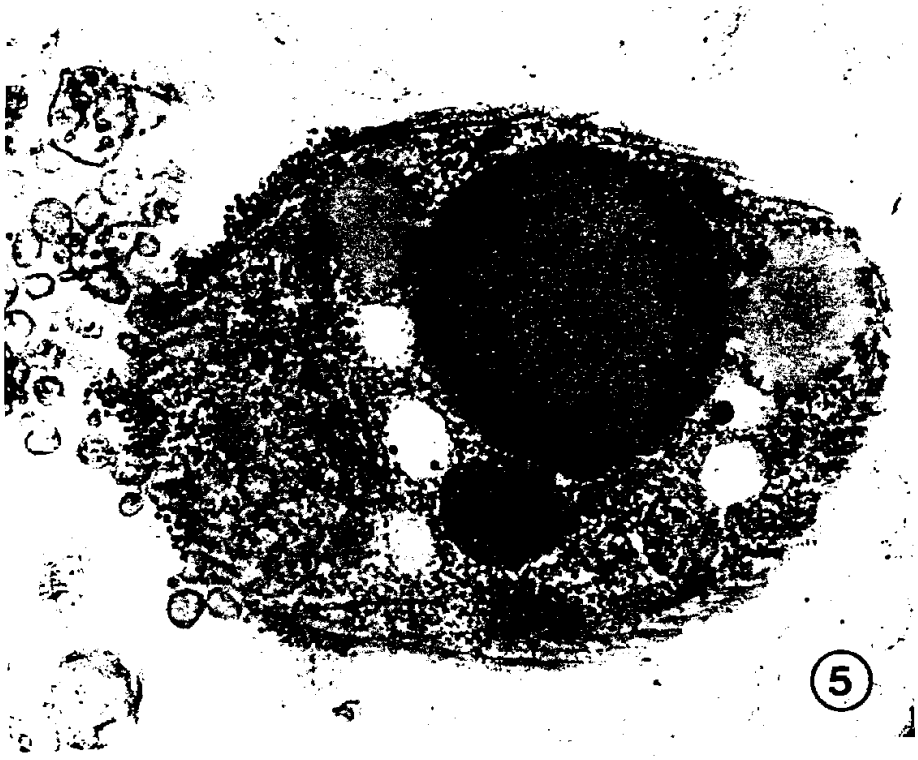

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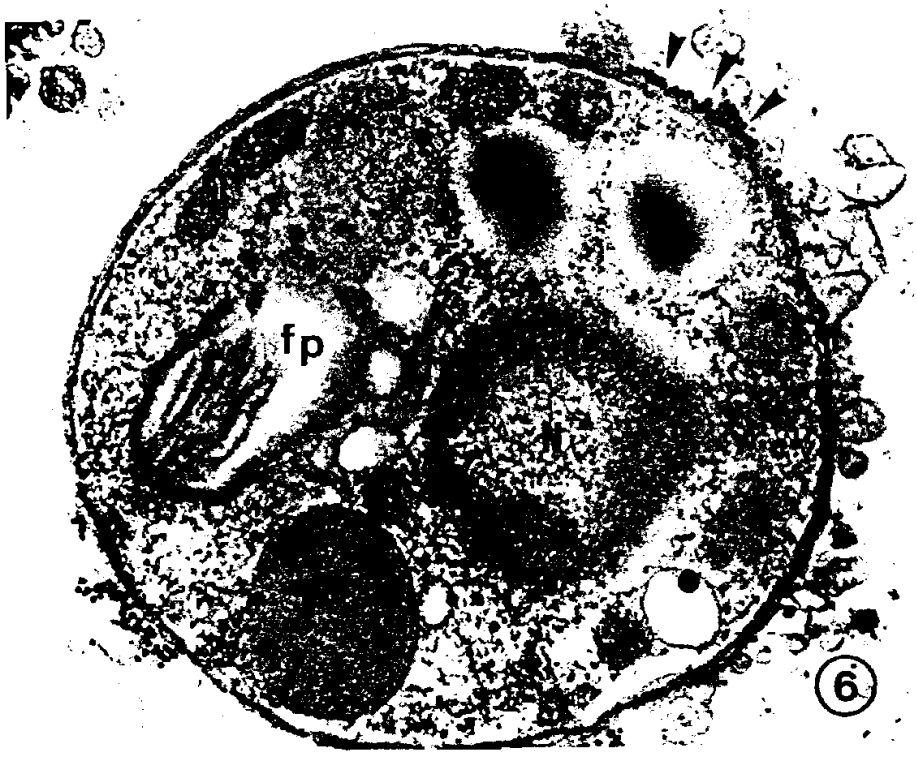

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the flagellar pocket in amastigote and promastigote stages of American leishmaniae, but only in the parasitic body close to the flagellar veil in the case of blood derived trypomastigotes. This contrasts to a diffuse distribution in cell culture derived trypomastigotes. The cellular basis for these differences remains to be established. It is important to mention that a patchy localization of laminin in plasma membranes has been previously demonstrated in mouse C1300 neuroblastoma cells (Liesi 1983) and in highly malignant, but not in poorly malignant, cells (McCoy *et al.* 1984).

The presence of laminin-like protein(s) of apparent parasitic origin on the plasma membrane of the members of the family Trypanosomatidae could explain the presence of antibodies to laminin reported in Chagas' disease (Szarfman *et al.* 1982), American cutaneous leishmaniasis (Avila *et al.* 1984), and in *T. rangeli* infected patients (Avila *et al.* 1986). Of importance is the finding that *Trypanosoma cruzi* trypomastigote laminin-like protein disappears rapidly once this parasitic stage begins to transform into the epimastigote form, suggesting that laminin-like protein could be a stage-specific protein, with a specific cellular function. As it is known that laminin mediates the attachment of epithelial cells to collagen type IV substrate (Terranova *et al.* 1980) and perhaps maintains cellular polarity in very early embryonic cells (Leivo *et al.* 1980), it is possible that laminin-like proteins could function in a similar manner in trypomastigotes, facilitating trypomastigote–host cell interaction or movement through small blood vessels. In the case of the amastigote stage of the genus *Leishmania,* the laminin-like protein could maintain the parasite bound to the phagolysosomal membrane via the opposite end of the flagellar pocket, as described recently (Bretaña *et al.* 1983), thus possibly favoring some specific physiological function.

Acknowledgments

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