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ISOLATION OF AN ENRICHED PLASMA MEMBRANE-SUBPELLICULAR MICROTUBULE FRACTION OF *LEISHMANIA MEXICANA AMAZONENSIS*

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A cell fractionation procedure previously developed for Trypanosoma cruzi *was applied to isolated the plasma membrane of promastigotes of* Leishania mexicana amazonensis. *The cells, swollen in an hypotonic medium, were disrupted in the presence of a nonionic detergent and the membrane fraction isolated by differential centrifugation. Electron microscopy showed that the fraction consisted of pieces of the plasma membrane associated with subpellicular microtubules. It was also shown that this fraction is able to induce cell-mediated immune response in mice.*

The cell surface of Trypanosomatidae can be considered as constituted by two components: the plasma membrane and a layer of microtubules, localized below it (subpellicular microtubules). High resolution electron micrography show the presence of a structure connecting the plasma membrane to the microtubules (Linder and Staehelin, 1977). Several studies have shown that carbohydrates are associated with the plasma membrane of trypanosomatids forming the glycocalix (De Souza and Meyer, 1976; Dwyer, et al., 1974). Since the cell surface of *Leishmania* may play role in the complex host-parasite interactions in the tegumentar leishmaniasis, attempts have been made to isolate the cell surface components of *Leishmania* applying the cell fractionation procedure recently developed for *Trypanosoma cruzi* (Pereira, *et al.*, 1978). The present work describes the results obtained applying the cell fractionation method above mentioned so as to obtain an enriched membrane-microtubule fraction from promastigotes of *L. mexicana amazonensis*. The morphology and immunogenic properties of the fraction are also described.

MATERIALS AND METHODS

Microorganism – The JMMO strain used in this study was isolated from a patient with diffuse cutaneous leishmaniasis during a period of treatment in the Hospital Santa

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Casa de Misericórdia in Rio de Janeiro, Brazil. The parasite has been maintained in hamster. This strain was classified as *Leishmania mexicana amazonensis.*

Cultivation – The parasite was cultivated in a diphasic medium with a rabbit blood agar base and a liquid phase of Brain Heart Infusion (BHI-Difco) for 6 days at 28°C. The parasites are usually maintained in culture by weekly transfer for a period no longer than 4 months. After cultivation for 6 days the cells were collected by centrifugation at 7500 g for 10 min and resuspended in buffered solution containing 100 mM NaCl, 20 mM K_2HPO_4 and 0.5 mM $MgCl_2$, pH 7.0.

Cell Fractionation – The fractionation procedure was carried out at 0-4°C and all isolation steps were monitored by using a phase contrast microscope. Unless indicated, the centrifugations were carried out in a Sorvall centrifuge model RC-2 with SS-34 rotor. After washing twice in buffered solution (composition given above) the cells were resuspended in an hypotonic medium containing 50 mM sucrose in 10 mM Tris-HCl, pH 7.5 (10 ml per gram of cells.) After 20min Lubrol PX (Sigma Chemical Company, USA) was added to a final concentration of 1% (v/v) and the cells disrupted with 30-40 strokes in a Dounce-type homogenizer (Kontes Glass, Vineland, N.J.), with a tight-fitting pestle. Isotonic conditions were restored by addition of 1 M sucrose to give a final concentration of 0.25 M sucrose. Two initial centrifugations at 700 g for 90 sec were made to separate the whole homogenate from large particles and also some non-disrupted cells. The homogenate was centrifuged twice at 5000 g for 10 min; the supernatant and two pellets were obtained. The supernatant was then centrifuged at 27000 g for 30 min and the resulting pellet designated as the membrane fraction.

Electron Microscopy – Intact cells and the membrane fraction were fixed in 2.5% glutaraldehyde in 0.1 M phosphate or cacodylate buffer, pH 7.4, for 2 hr at room temperature. After rinsing in 0.25 M sucrose in 0.1 M phosphate buffer, they were post-fixed for 2 hr at 4°C with 1% OsO_4 solution in 0.1 M phosphate buffer, washed, dehydrated in acetone and embedded in Epon. Ultrathin sections were cut with an LKB Ultratome III ultramicrotome, collected on copper grids and examined, after staining with uranyl acetate and lead citrate, in an AEI EM6-B electron microscope.

Immunization of Mice – Female swiss albino mice of 20-25 g were used. Immunization with the membrane fraction was carried out in 3 groups of 10 mice using different schedules of vaccination, as shown in Table 1. A control group of 10 mice received similar schedule of injections without the immunogen. Seven days after the last injection all animals were subjected to immunological studies.

Agarose Gel Diffusion – The presence of precipitating antibodies was determined by double diffusion in plates containing 1.5% agarose. Membrane fraction in different concentrations was assayed against undiluted mice sera (Avrameas et al., 1969).

Passive Hemagglutination – Erythrocytes sensitized to membrane fraction by glutaraldehyde (Avrameas et al., 1969) and the titration of antiserum was carried out in microtiter plates.

Plaque forming cells – Direct plaque forming cells (PFC) to SRBC was carried out by the method of Cunninghan and Szenberg (1968) in suspension of spleen cells obtained 4 days after intravenous injection of 0,2 ml containing 1×10^8 SRBC. The animals were studied 7 days after the last immunizing injection of the membrane fraction. Results.were expressed by the number of PFC per 1×10^6 spleen cells.

Footpad test – For the determination of mice delayed type hypersensitivity 20 μl of membrane extract, containing 12 μg protein, were injected, intradermally, in one footpad of immunized and control mice. An equal volume of saline was injected in

TABLE I

Schedules of immunization with the membrane fraction isolated from *Leishmania mexicana amazonensis*. The different groups of animals were studied 7 days after the last immunizing dose.

Group (10 Animals)	*Immunization with the Membrane Fraction*				*Total Dose (μg Protein)*
	Days	*0*	*7*	*14*	*21*
FM_1		50μg+FIA (f.p.)	50μg+FIA (f.p.)	100μg+Saline (s.c.)	100μg+Saline (s.c.)	300 μg
FM_2		25μg+FIA (f.p.)	25μg+FIA (f.p.)	50μg+Saline (s.c.)	50ug+Saline (s.c.)	150μg
FM_3		50μg+Saline (f.p.)	50μg+Saline (f.p.)	100μg+Saline (s.c.)	100μg+Saline (s.c.)	300μg
Control		Saline+FIA (f.p.)	Saline+FIA (f.p.)	Saline (s.c.)	Saline (s.c.)	–

FIA: Freund Incomplete Adjuvant; sc: subcutaneous; f.p. footpad.

the other footpad. Results were expressed as the difference in footpad thickness between test and control, measured after 24 hr with a micrometer caliper.

Blastogenic response of spleen cells to mitogens – The ability of cultured spleen cells from normal and immunized mice to proliferate in response to stimulation *in vitro* with PHA (PHA-M, Difco), Con A (Concanavalin A, Sigma) and LPS (Lipopolysaccharide *S. thiphy*, Difco) was determined by incorporation of tritium-labeled thymidine as recomended by Moorhead (1978). The percentage of inhibition was calculated by the formula:

$$\% \text{ inhibition} = 1 - \left\{ \frac{\text{CPM stimulated} - \text{CPM unstimulated (experimental)}}{\text{CPM stimulated} - \text{CPM unstimulated (control)}} \right\} \times 100$$

Leukocyte adherence inhibition (LAI) – Direct LAI assay was carried out according to Powell, Sloss & Smith, (1978). The mixture containing 1×10^7 spleen cells and 25 μg of membrane fraction protein was incubated at 37°C for 30 min. The LAI value is the difference between the means percentage of adherent cells of control and test sample, divided by the control value.

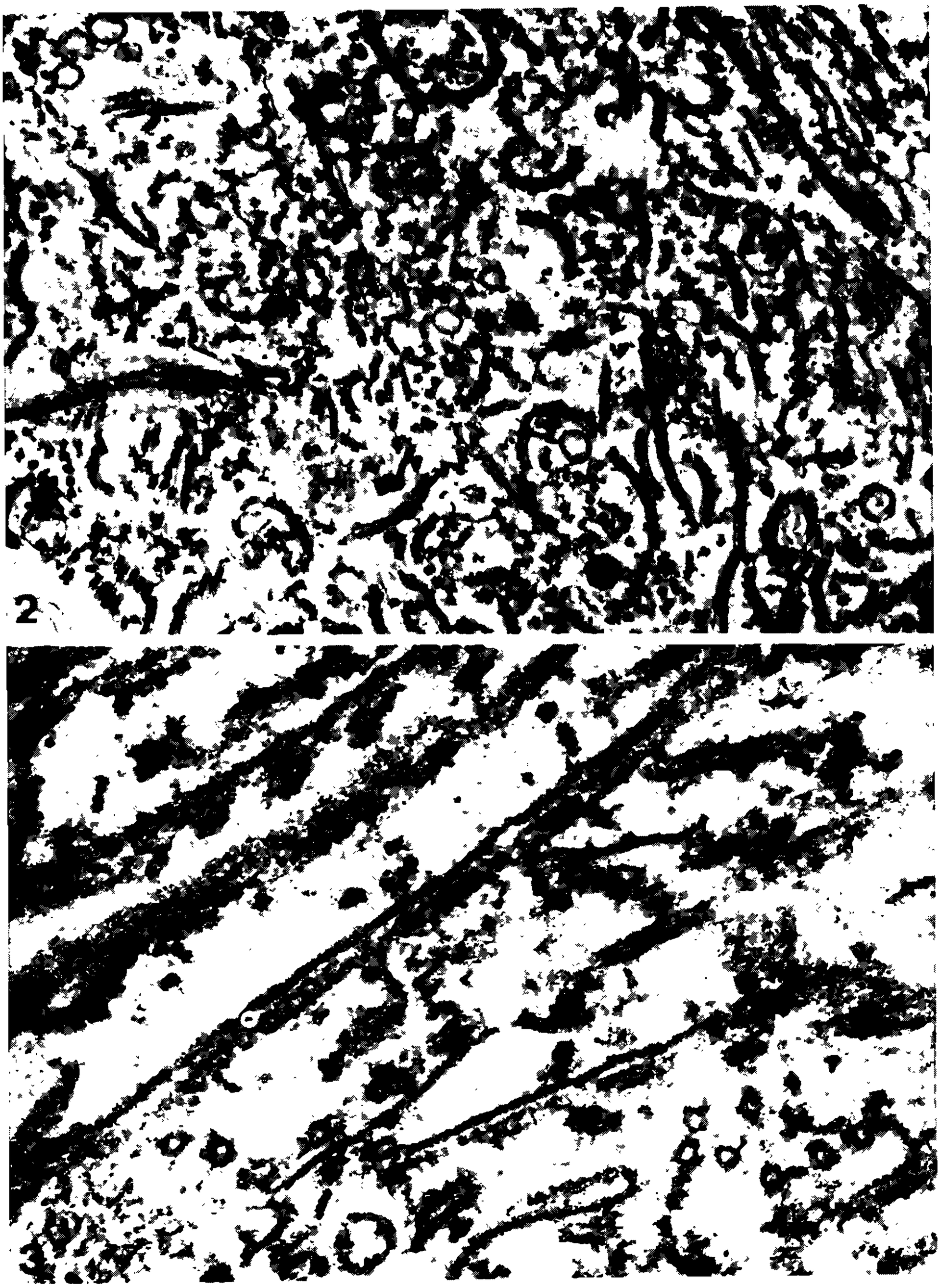
RESULTS AND DISCUSSION

As observed in all trypanosomatids, the cell surface of *Leishmania mexicana amazonensis* has an unit membrane and below it, a layer of subpellicular microtubules which are longitudinally oriented in relation to the anterior-posterior axis of the cell. They are separated from the inner leaflet of the plasma membrane by a space about 9 nm and from each other by a space of about 20 nm. The latter distance is maintained constant in any region of the cell.

The examination of the membrane fraction by electron microscopy showed that it consists of pieces of the plasma membrane associated with the subpellicular microtubules (Fig. 1). In tangential of oblique sections showing both the plasma membrane and the subpellicular microtubules we could observe that the distance between these two components of the cell surface of the protozoa maintains the same relationship found in intact cells, eg, the distance between them did not change (Figs. 2-3). Close association between plasma membrane and subpellicular microtubules have been previously observed in promastigotes of *Leptomonas collosoma* (Hunt and Ellar, 1974; Linder and Staehelin, 1977), and epimastigotes of *Trypanosoma cruzi* (De Souza, 1976). These results point out the existence of a close association between membrane and microtubules and is possible, as previously suggested (Meyer and De Souza, 1976) that this complex plays a role in the change of the form of the cell when a developmental stage changes into another. Indeed *Leishmania* species alter considerably their form when the rounded intracellular amastigote (spheromastigote) transforms into the longer promastigote.

The same method we used in this study was also applied to isolate plasma membrane of epimastigotes of *T. cruzi* (Pereira et al., 1978). However, the isolated membrane fraction consisted of empty closed vesicles with a diameter varying between 0.2 and 0.9 μm. Microtubules were not seen associated with the membrane. Similar result was also described by Segura et al. (1977). Since association of microtubules to the plasma membrane was observed in epimastigotes of *T. cruzi* lysed by osmotic shock (De Souza, 1976) it seems to us that the absence or presence of this association in cellular fractions is probably influenced by the method used to rupture the cells. However, we cannot exclude the possibility that this association varies according to the genus, species or even, the de-

Figure 1. General view of the plasma membrane subpellicular microtubule fraction of *Leishmania mexicana amazonensis.* X 9.000.



Figures 2 and 3. Transversal and longitudinal sections through the plasma membrane subpellicular microtubule complex of *Leishmania mexicana amazonensis*. The distance between the microtubule and the plasma membrane as well as between each microtubule remains the same observed in intact cells. Fig. 2: X 15.000; Fig. 3. X 120.000.

velopmental stage of trypanosomatid. Indeed, with the same technique (use of the detergent Lubrol PX) microtubules were not found to be associated with the plasma membrane of *T. cruzi* while they were associated with the membrane of *L. mexicana amazonensis*.

The study of purified cell fractions can tell us much about the morphology, biochemistry and immunological properties of specific cellular structures. These structures may change in their properties during the course of the life cycle of the parasites. The cell surface, in particular, might be expected to show changes as it lies at the host-parasite interface. Indeed, antigenic and immunological changes have been described during the amastigote-promastigote transformation in *Leishmania donovani* (Simpson, 1968; Dwyer, 1976) and *L. braziliensis* (Dawidowicz, Hernandez & Infante, 1975). It is possible that these differences are in part related to cell surface properties. A comparative biochemical analysis of isolated membranes of both developmental stages may aid in explaining these differences. The membrane-microtubule fraction can also be used in studies related with properties of the microtubules of trypanosomatids, the nature of the association between plasma membrane and microtubules, and immunological properties.

The results obtained in immunization of mice with the membrane fraction, using different schedules of vaccination (Table I), showed that this fraction was capable of inducing an expressive cellular immune response as revealed by the results of footpad test (Table II) and leukocyte adherence inhibition (Table III). However it was unable to evoke a similar humoral response. Specific antibodies induced by this fraction, in all groups of mice, were absent or in very low titer, as detected by gel diffusion and passive hemagglutination, even when the immunogen was injected emmulsified in an adjuvant (Table IV). The possibility that our membrane fraction was endowed with the property of inducing some degree of suppression of B lymphocytes was excluded. This question was ascertained by the ^{3}H-thymidine incorporation by these cells, stimulated by Con A and LPS (Table V) and by the number of direct PFC, procuded *in vitro* by spleen cells of mice, intravenously sensitized to SRBC (Table VI). According to our results, the membrane fraction obtained as described in this paper seems to be a good immunogen in evoking a cell-mediated immune response.

TABLE II

Delayed type hypersensitivity of mice 24 hr after footpad injection of 20 μl containing 12 μg of protein from the leishmanial membrane fraction. Tests performed 7 days after the last immunizing dose of the antigen.

Groups (10 Animals)	*Footpad Swelling (mm) After 24 hr*	*P Value*
FM_1	0.41 ± 0.25	< 0.02
FM_2	0.36 ± 0.28	< 0.05
FM_3	0.22 ± 0.09	< 0.05
Control	0.04 ± 0.03	–

TABLE III

Adherence inhibition of spleen cells of mice sensitized to leishmanial membrane fraction. Experiments performed with 1×10^7 spleen cells in the presence of membrane fraction containing 25 μg of protein.

Groups (10 Animals)	*Leucocyte Adherence Inhibition % ± SD*	*P Value*
FM_1	93 ± 5.8	< 0.001
FM_2	76 ± 10.0	< 0.001
FM_3	46 ± 15.0	< 0.001
Control	19 ± 9.0	–

TABLE IV

Serologic response of mice submitted to different schedules of immunization with leishmanial membrane fraction.

Animals		*Precipitation in Gel Diffusion*	*Passive Hemagglutination*
Nº	*Groups*		
10	FM_1	0	1:2 (6)* 1:4 (2) 1:8 (2)
10	FM_2	0	1:2 (5) 1:4 (1) 1:8 (4)
10	FM_3	0	1:2 (4) 1:4 (3) 1:8 (2) 1:16 (1)
10	Control	0	0

*Number in parenthesis are of animals giving positive reactions.

TABLE V

(^{3}H) – thymidine incorporation by 1×10^6 spleen cells in the presence of Con A (5 μg/ml), LPS (100 μg/ml), leishmanial membrane fraction (50 μg/ml). Cells from mice subjected to different schedules of immunization with the leishmanial fraction. Stimulation index was calculated by dividing the CPM obtained in the presence of antigen by CPM without antigen.

Groups (5 Animals)	*Con A 5 μg/ml*		*Ag 50 μg/ml*		*LPS 100 μg/ml*	
	Stimulation index	% *Inhibition*	*Stimulation index*	% *Inhibition*	*Stimulation index*	% *Inhibition*
FM_1	12.0	0 (14.0 ↑)	3.4	0 (126 ↑)	3.8	24.5
FM_2	11.5	0 (9.5 ↑)	3.0	0 (100 ↑)	2.2	56.0
FM_3	13.8	0 (31.4 ↑)	1.8	0 (20 ↑)	8.8	0 (76 ↑)
Control	10.5	–	1.5	–	5.0	–

↑ % of stimulation.

TABLE VI

Hemolytic plaque formation (IgM) by 1×10^6 mice spleen cells, 4 days after the i.v. injection of 1×10^8 SRBC. The animals were previously immunized to membrane fraction of *Leishamania mexicana amazonensis* by different schedules of vaccination. Absence of immunossupressive effect on B lymphocytes.

Group (10 Animals)	*Plaque Forming Cells 1×10^6 Cells ± SD*	*P Value*
FM_1	1.528 ± 390	< 0.02
FM_2	1.475 ± 523	< 0.10
FM_3	1.510 ± 540	< 0.10
Control	1.055 ± 143	—

RESUMO

Um método de fracionamento subcelular, previamente desenvolvido para *Trypanosoma cruzi*, foi aplicado para isolar a membrana plasmática de promastigotas de *Leishmania mexicana amazonensis*. As células, após turgimento em meio hipotônico, foram rompidas na presença de um detergente não iônico e a fração de membrana isolada por centrifugação diferencial. A microscopia eletrônica mostrou consistir a fração de fragmentos de membrana plasmática associados com microtúbulos subpeliculares. Foi também mostrado que esta fração era capaz de induzir resposta celular em camundongos.

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