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Atomic Force Microscopy of host cell-amastigote interaction in cutaneous *leishmaniasis*

Interacción amastigote-célula en leishmaniasis cutánea por Microscopía de Fuerza Atómica

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Abstract

Natural infection produced by the entry of promastigotes into the skin cells induces cutaneous leishmaniasis disease and the Interaction between phagocytic cells and different strains of Leishmania determine the course of disease in the mammalian host. In this study the cutaneous lesions from hamsters infected with the L. garnhami were pressed on a microscope slide, allowed to dry and methanol-fixed in order to be ultrastructurally analyzed using atomic force microscopy (AFM). Free amastigotes showed their nucleus, kinetoplast and a depression of 5 microns, corresponding to the refractil organelle characteristic of this Leishmania. Parasites inside macrophages and lymphocytes and the topographical relationship with the host cytoplasm was also observed. Parasite-host-cell interaction revealed different membrane contact. The amastigote-macrophage contact is established by small macrophage filopodio associated with its cytoplasmic reduction near the contact site of 800 nm. The amastigote-lymphocyte contact shows fusion-like behavior between the two membranes without any cell specialization. The cutaneous lesion studied with AFM allowed observation with high resolution the close contact established between the parasite and its host cells as well as details of the fine structure of the amastigotes, applying a simple and rapid tissue preparation.

Key words: Atomic force microscopy, Leishmania garnhami, lesiones cutáneas, amastigote, macrophage, lymphocyte, 3D image.

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Resumen

La infección natural producida por la entrada de promastigotes en la piel produce leishmaniasis cutánea y la interacción entre las células fagocíticas y las diferentes cepas de Leishmania determina el curso de la enfermedad en el hospedador vertebrado. En este estudio las lesiones cutáneas de hamsteres infectados con L. garnhami fueron frotadas sobre un portaobjeto, secadas a temperatura ambiente y fijadas con metanol para analizarlas ultraestructuralmente usando un microscopio de fuerza atómica (AFM). Los amastigotes mostraron el núcleo, kinetoplasto y una depresión de 5µ, correspondiente a la organela refráctil característica de esta especie. Los parásitos dentro de los macrófagos y de linfocitos y las relaciones topográficas con el citoplasma de la célula hospedadora, revelaron diferentes tipos de contacto entre las membranas celulares. El contacto amastigote-macrófago se establece por un pequeño filopodio del macrófago asociado con reducción citoplasmática cerca del sitio de contacto de 800 nm. El conctato amastigote-linfocito mostró estrecha fusión entre las dos membranas sin ninguna especialización celular. Concluimos que las lesiones cutánes estudiadas con AFM permite la observación con alta resolución, del estrecho contacto que se establece entre los parasites y sus células hospedadoras así como, los detalles de la ultraestructura de los amastigotes aplicando una simple y rápida preparación de las muestras de tejido.

Palabras clave: Microscopía de fuerza atómica, Leishmania garnhami, lesiones cutáneas, amastigotes, macrófagos, linfocitos, imagen 3D.

Introduction

Cutaneous leishmaniasis is a significant cause of morbility in several countries of the world. Leishmania parasites are intracellular protozoa transmitted by the bites of sand fly species, producing clinical manifestations which may vary from simple cutaneous lesions to mucocutaneous ulcers or visceral diseases (27). The diseases caused by all species of the genus Leishmania are dependent on the fact that these parasites multiply and survive in the mononuclear phagocytes of the skin in cutaneous leishmaniasis (2).

Parasite L. garnhami produces cutaneous lesions among people living in urban and rural areas in Mérida State, Venezuela and possesses a particular organelle that has been seen as a refractil organelle (19). The biometrical atributes of the amastigote stage by light microscopy showed that this parasite has a range of 3.16 to 3.95 µ in length, the nuclear and kinetoplastic indexes ranged respectively from 0.41 to 0.56 and 0.64 to 1.07 µ fitting into the values of amastigotes of the mexicana group (14).

The entry of these parasites into the skin cells induce interactions between phagocytic cells and Leishmania, producing dermal lesions in man and domestic mammals such as donkeys and dogs, and have been searched for in the skin, viscera and blood of most of the mammalian host suspected to be reservoirs as a parasite source for sandflies (11).

The purpose of this research was direct visualization of Leishmania amastigotes in mononuclear phagocytes in skin biopsy samples of experimental animals infected with L. garnhami. To analyzed high-resolution images and details of the surface structure of parasite-host cell interaction at a resolution of a few nanometers, using an atomic force microscopy technique (AFM), can be achieved for studies of light microscopy (LM).

It Is an essential tool for imaging surfaces in applications in cell biology and bioma-

terial science, measuring surface topography on a scale from angstroms to 100 microns. It can be used to measure the attractive or repulsive forces between the sample surfaces, revealing chemical and mechanical properties like adhesion and elasticity. Recently, this technique has been applied widely in the analysis of human sperm and membrana of human erytrocytes (9, 10, 26).

In this report, we document the integration the AFM with LM by showing the intracellular location and contact between parasites and phagocytic cells.

Material and Methods

Animales experimentales

A total of five golden hamster males (Mesocricetus auratus), 7 weeks old, were obtained from the Laboratory colony (BIOULA) at the Universidad de los Andes, Merida, Venezuela.

Inoculation experimental

The leishmanial strain used for this study, L. garnhami H/HOM/Ve/JAP-76 (19), was grown in the bifasic culture NNN and incubated at 26°C.

Promastigotes were harvested from the liquid overlay of NNN medium after 10-15 days, and counted using a Neubauer hemocytometer. Serial dilutions were made in PBS pH 7,2 to obtain 50 promastigotes/.L Hamsters were inoculated intradermally in the footpads with 5.104 promastigotes contained in a volumen of 0.05 ml of saline solution. All the animals were maintained at 23°C with a comercial diet, ratarina,R and water ad libitum until they were sampled 9 week post-innoculation (pi).

Preparation of the skin samples

The animals were killed with ether 9 weeks pi, and the skin biopsis samples were taken from lesions in the rear hind leg, the tissue samples were pressed on a microscope slide, methanol-fixed to 5°C and allowed to dry. Microscopic preparations of the skin lesions of hamsters infected with L. garnhami were ultrastructurally analyzed. In this study an atomic force microscope unit attached to a conventional Olympus microscope (BX 60) was used.

Results

In this research we used this approach for investigating the structure near the upper surface of the membrane of the amastigotes in skin lesion, and reconstruction of three dimensional (3D) images employing a AFM procedure. It presents a wide selection of high-resolution images in simple fixed smear skin biopsies of the cutaneous lesions that are not possible to show by optical microscopy.

At low magnification details of free amastigotes as well as the variation of the amastigotes shape could be distinguished only. At higher magnification the parasite nucleus measured 1803 nm in length and a depression in the cytoplasm was found to be of 206 nm (Figure 1 A, B, C, C').

Examination of numerous skin samples revealed amastigotes inside of macrophage and one of them measured 4868 nm (Figure 2 A, B, B'). The interaction between Leishmania and macrophages was observed across of filopodia at the contact site. In this area the macrophage cytoplasm is less compact and the filopodia measured about 813 nm (Figure 3 A, B, C, C', D). The three-dimensional topographic images corresponding to the parasite-macrophage contact site is showed in Figure 3 D'.

AFM techniques used on impression smears of hamster cutaneous lesions, revealed Leishmania-lymphocyte interaction. The lymphocytes make the initial contact with almost the whole length of the parasite and the

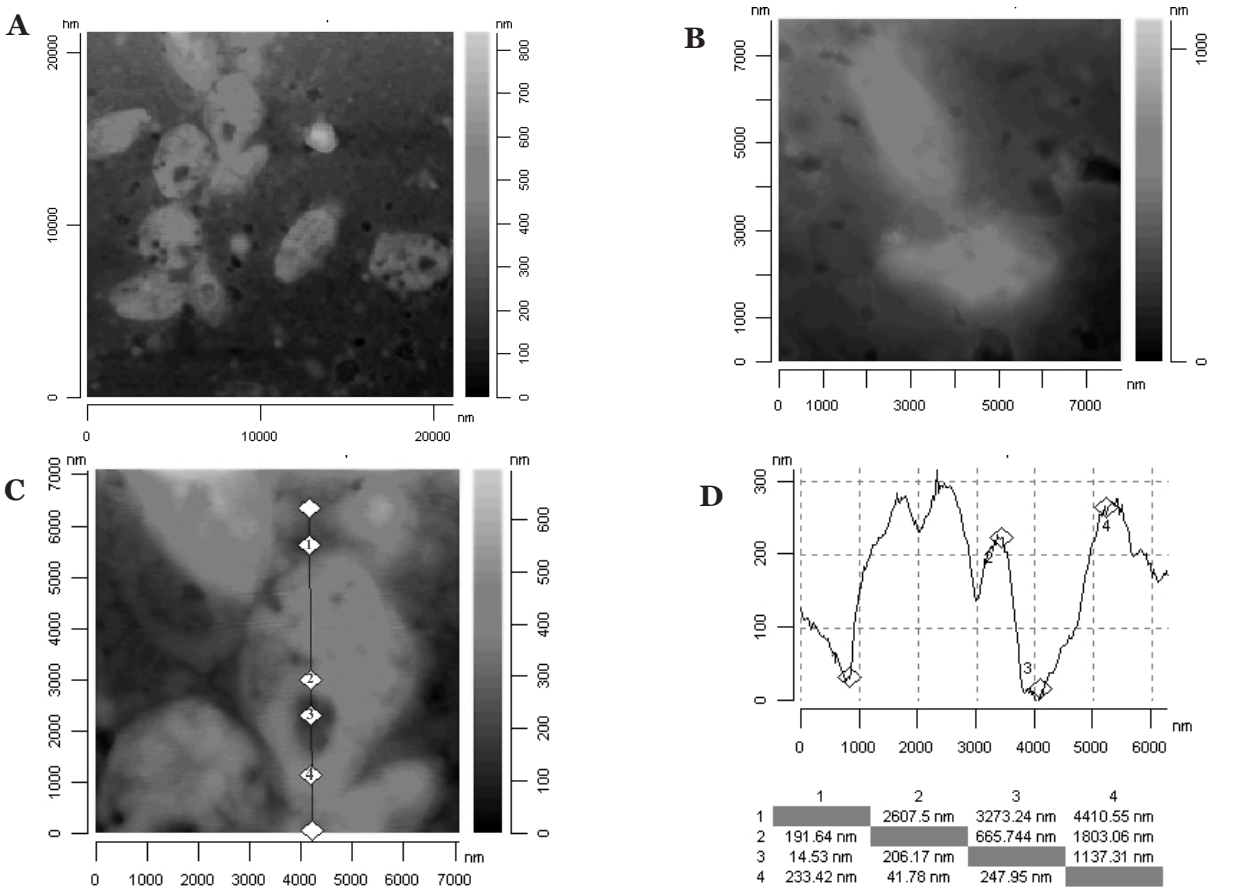

Figure 1. shows the ultrastructural details of free amastigotes obtained by AFM. At low magnification (**A**) the variation of the amastigotes shape can be distinguished. (**B**) The nucleus (**N**) and the kinetoplast (**K**) are observed, at higher magnification (**C**), the nucleus is observed as well as a depression in the cytoplasm and it corresponds to the refractile organelle characteristic of this *Leishmania*. (**C'**) shows that the nucleus measured 1803 nm in length (points 2-4) and the depression was found to be of 206 nm. (points 2-3).

filopodia appeared at the contact site. In this site the parasite width was 2644 nm and its height was 379 nm (Figure 4 A, A', B, B', C).

Three-dimensional representation of the AFM images showed the amastigote cytoplasm located on top of the slender lymphocyte cytoplasm (Figure 4 C').

Discussion

Identification of the biological structures is often problematic in scanning probe microscopy (18). Only when the studied specimen has a recognizable shape, such as blood cells, or when it contains periodic information, is the identification easy.

American cutaneous leishmaniasis that present a non-specific chronic inflammation and specific diagnosis is possible only by finding the parasite in the tissues. The parasites are usually few in number and difficult to find in some skin or mucosal lesions or in unusual localizations, and are not diagnostic by classical microscopic examination (15, 16).

The use of the AFM technique is appropriate to observed amastigotes and combinations of one or more patterns of antigen presentation in the same biopsy, partially processed as for the routine light microscope and screening of cutaneous leishmaniasis, as well as details of the fine structure of the amastigotes and its host cells.

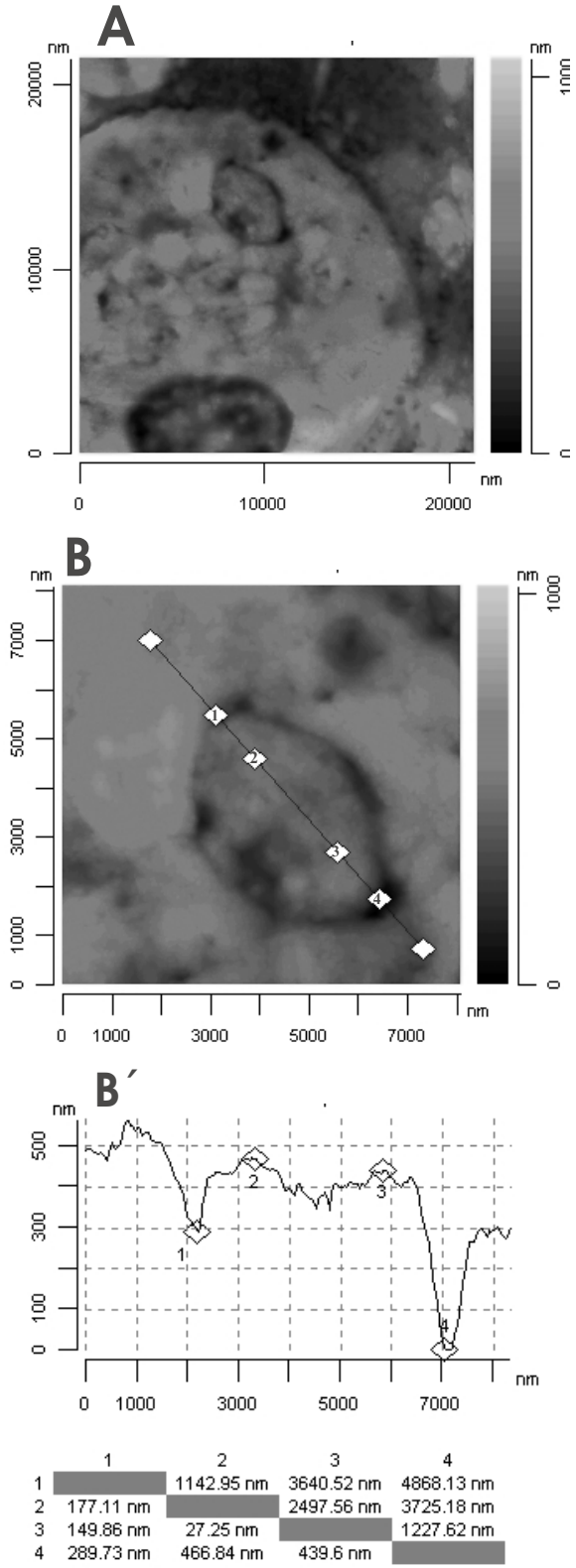

Figure 2. shows the image obtained of parasite of *Leishmania* **(A)** inside a macrophage. **(B).** Notice that the parasite measured 4868 nm (points 1-4 in **B'**).

AFM method should be used in several specimens collected from tissue from different organs with inflammatory processes produced by Leishmania, dispersion of parasites by lymphatic route, as well as to show distribution patterns at the site of the inoculation, and studied in conjunction with clinical signs, laboratory data with special emphasis on histomorphological findings in the skin in order to determine a diagnosis.

On the other hand, future studies will include analysis of the treatment of cutaneous lesions and of clinical response to pentavalent antimonials and persistance of viable amastigotes inside the tissue, on the basic of the reactions of susceptible hosts to primary contact with Leishmania and specific phagocytic functions of macrophages (11, 20).The presence of amastigotes on smears of peripheral blood was shown in circulanting blood cells as occur in saurian Leishmania species as well as occupation of thrombocyte (24).

Giemsa stained smears show rare intramonocytic inclusions suggestive of leishmanias, Several heavily infected monocytes present on the buffy coat, bone marrow aspirate show the presence of intramonocytoplasmic leishmanias.

In histological preparations the amastigotes had been observed inside of peripheral blood leucocytes of patients with mucosal disease (13). Peripheral intramonocytic leishmanias in blood smears of AIDS patient infected by L. donovani (8) were also observed.

On the other hand, Leishmania (Viannia) braziliensis was observed on the vascular endothelium lesions, free in the capillary lumen and cytoplasm neutrophils of the skin lesions of patient with cutaneous leishmaniasis (12).

Perhaps the only characteristic finding on examination of the smears Giemsa-stai-

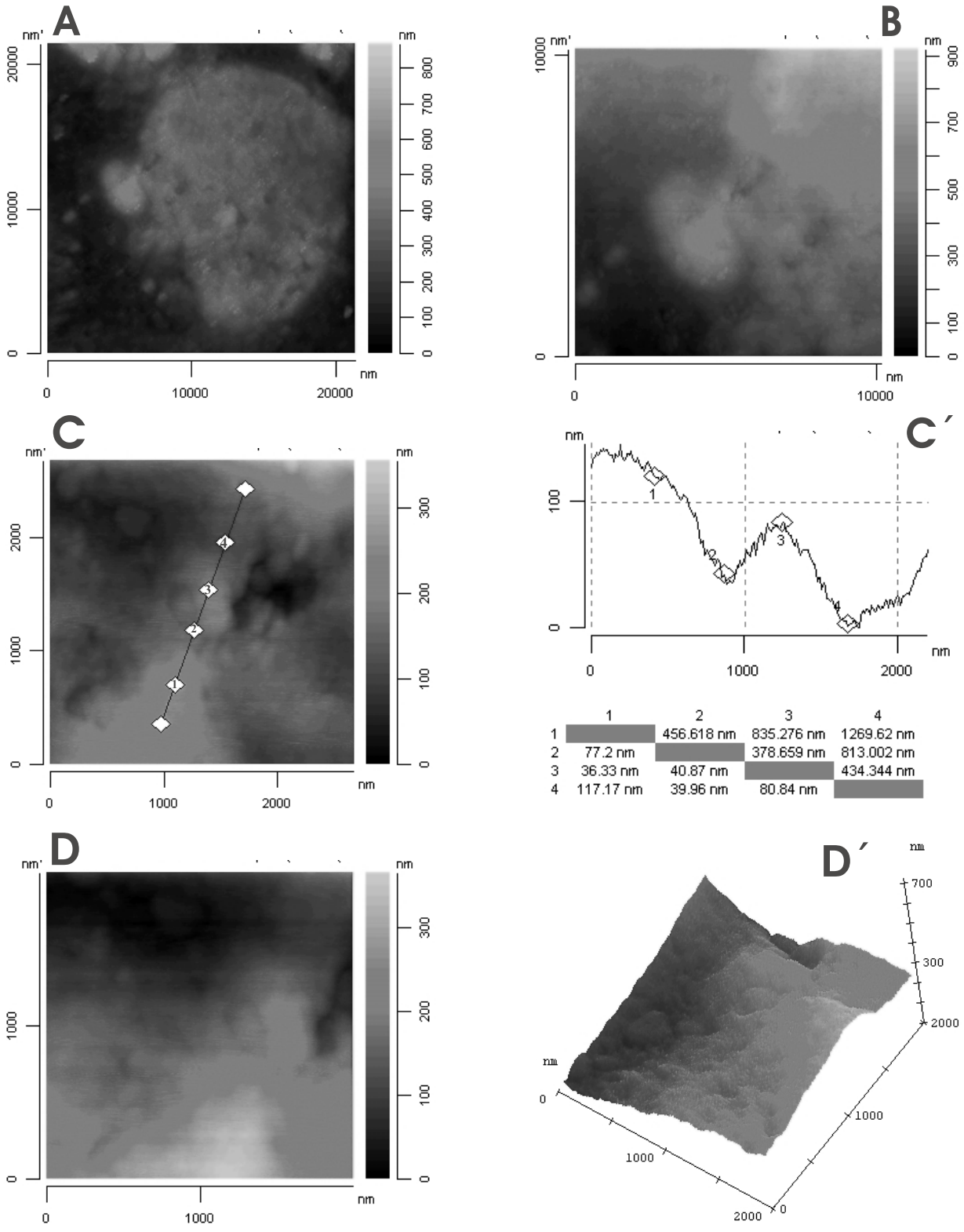

Figure 3. clearly shows the *Leishmania*-Macrophage interaction. Low magnification shows a parasite making contact with the cytoplasm of a macrophage (**A**). At higher magnification (**B**) filopodia appeared to be present at the contact site (**C**). Notice that the macrophage cytoplasm near the contact site is less compact and the filopodia measured about 813 nm (distance between points 2 and 4 in **C'**). Figure **D'** shows a detail of the topography in 3D of the parasite-macrophage contact site **(D)**.

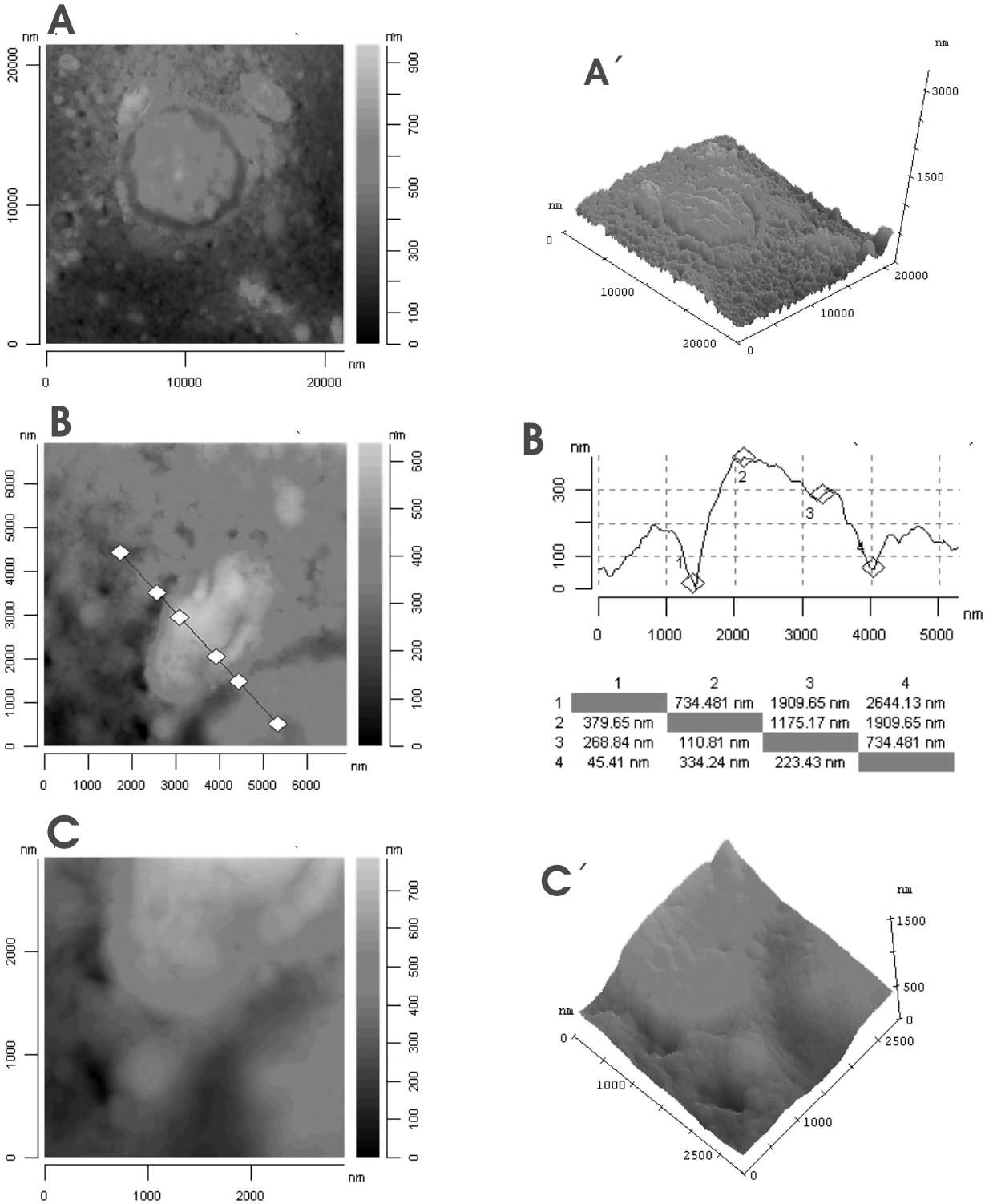

Figure 4. *Leishmania*-lymphocyte interaction: (**A**) Low magnification that shows two parasites in contact with a lymphocyte. (**A'**) a 3D image of figure A. The lymphocyte makes the initial contact with the almost the whole length of the parasite as seen in Fig. **B**. Notice that the kinetoplast is making contact with the lymphocyte cytoplasm (asterisk). and the filopodia appeared to be present at the contact site (**C**). The parasite width is 2644 nm (points 1-4) and its height is 379 nm (points 1-2) in **(B')**. Fine detail of the parasite-lymphocyte contact (**C**): The 3D image processing (**C'**) allows to show that the parasite cytoplasm is located on top of the slender lymphocyte cytoplasm.

ned skin biopsies, is the observation of amastigotes, macrophages and inflammatory cells regularly dispersed on the slide, but not details related with their cell surface and membrane interaction. Other authors also proposed that AFM technique can be used to measure binding properties of biological systems, specific interaction between two kinds of molecules as well as the specific antigen-antibody interaction, in small preparations and show signal electronic signals in a three dimensional map of the cellular surface (5, 17, 25). In other tropical disease parasites have been detected by introducing the ultrastructure study of all the parasite developmental stages (6, 7).

The use of different microscopic techniques such as light, electrón, and confocal microscopy, are powerfuls tools to answer question and to solve problems both in biology as well as in material science.

In this study mastigote-phagocytic cell interaction was observed and it is probable that the positive charge of the parasites at the side of contact ficilitates binding of cationic particles with host cell (23, 1).

There is evidence that the process of interaction of Trypanosoma cruzi with the host cell can be considered as one involving ligands and receptors (3), most probably immediately after the initial parasite contact, a change takes place in the plasma membrane of both cells, it has been shown that parasite proteases, is involved in this process (22). In some cells such as macrophages, cell surface projections arise, resembling those typically observed during phagocytosis and it is clear that following parasite attachment there is a process of Ca2+ release from both the parasite and the host cell (4).

The process of interaction of amastigote forms of L. garnhami with other cells could be associated with the distribution of the actin filament of the host cell, as was evaluated by visualization of the filaments of cells marked cytochalasin D using confocal laser scanning microscopy, which interfered with the distribution of actin filaments during the process of internalization of the trypomastigote forms into the host cells (21). In fact, in the present work the consistent ultrastructural observation of small macrophage filopodio revealed a subsecuent reduction of the cytoplasm at the contact site.

Conclusions

The morphology of the lesions in the nodules has been studied, both in human and in experimental models. Certain infected animals show few or no parasites, and are infiltrated by mononuclear cells surrounded by large bundles of collagen fibers and plasma cells. In our study of cutaneous lesions most of the intracellular parasites show a normal ultrastructure, and host cell cytoplasm close to these adherence sites becomes vacuolated. The discovery of leishmania amastigotes when examining blood smears or histological samples, is an important clue to diagnosis in particular circumstances, which is probably indicative of low parasite density. The diagnosis of post Kala-azar dermal leishmaniasis can be made clinically in patient who has characteristic manifestations, however, it can be confused clinically and pathologically with other dermatoses, particularly leprosy (27).

The findings in this report confirm and extend those of previous studies on macrophage-Leishmania interaction and we believe that with AFM systems provides a more relevant diagnostic method to analyze host-parasite interactions, as well as the analysis of lymphokine-induced alterations in macrophages-parasite interactions. In cutaneous leishmaniasis some macrophages and

epidermal keratinocytes contained Leishmania antigen only, without parasites or were useful in the screening of the disease in epidemiological surveys, in immuno-compromised patients, and that it is possible to fortuitously discover intramonocytic leishmanias when performing routine leukocyte differencials (8). We recommended adopt the AFM practice in the biomedical field to obtained ultramorphological images and details in the surface structure of cells and organelles having very irregular and complicated surfaces or when parasites are not observed for diagnosis and characterization of the Leishmania species with a scanning probe microscopes in samples. This does not have not to be specially prepared.

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