

Is the Amastigote Form of *Leishmania* the Only Form Found in Humans Infected With Cutaneous Leishmaniasis?

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Abstract

Background: Previous studies of *Leishmania* revealed that 2 forms of the parasite are present: the intracellular amastigote form found in the vertebrate host, and the promastigote form predominately found in the insect vector.

Methods: Samples were collected from the lesion area of the skin of 42 patients referred to the laboratory and microscopic slides were prepared.

Results: Out of 42 cases, only 1 (2.4%) showed the presence of the amastigote intracellularly alone but not in the extracellular

area, and 2 (4.75%) showed the presence of the amastigote in the extracellular but not in the intracellular location. In 20 of 42 cases (48%), we observed the amastigote presence in the intracellular or extracellular area. Out of all 20 cases in which the amastigote was recovered, we were able to identify the amastigote in 17 cases (85%) in the intracellular and extracellular locations simultaneously. Additionally, out of all 42 cases referred, the amastigote form alone, with no promastigote associated, was microscopically recovered in 10 cases (24%).

Conclusion: Contrary to previous studies, we found that in the human body *Leishmania* does

not act as an obligatory intracellular parasite. *Leishmania* is an intracellular and extracellular parasite infecting the mononuclear phagocyte first. There, it remains in the form of amastigote and multiplies by binary fission. When released from the mononuclear phagocyte, after membrane rupture to the extracellular fluid, the amastigote transforms into a promastigote-like form in a progressive sequence. At a later stage, this promastigote-like organism continues its development and transforms again to produce pseudofiber, leaving the lesion area with a permanent scar.

In 1997, we received the first challenging specimen referred from a consultant dermatologist at a hospital belonging to the Ministry of Health in Saudi Arabia for cutaneous leishmaniasis identification by the microscopic smear method. Clinically, the lesion looked typical for the dried type of cutaneous leishmaniasis with a painless ulcer and a raised, indurated margin and a necrotic base. On performing the general method for microscopic exam and staining with Wright stain, we were unable to identify the Leishman-Donovan (LD) bodies within the macrophages, which is the principle of the microscopic diagnosis of cutaneous leishmaniasis. Afterwards, for the next 10 years in practice, we had several similar cases, and we failed to demonstrate the amastigote presence in approximately 50% of the cases by microscopic smear.

The purpose of our study was to microscopically identify any extracellular form of *Leishmania* present in human lesions and, if found, to describe the new form microscopically and its transformation sequence on a step-by-step basis with photo support.

Literature Review

The group of diseases known as the leishmaniasis are caused by protozoa of the genus *Leishmania*. These are present in 3 different forms: visceral leishmaniasis (VL), mucocutaneous leishmaniasis (ML), and cutaneous leishmaniasis (CL). The visceral form (kala-azar) is the most severe form of the disease and, if left untreated, is usually fatal. Mucocutaneous leishmaniasis is caused by *Leishmania braziliensis* or related New World species, and parasites may disseminate to the oral and nasopharyngeal mucosa.

Cutaneous leishmaniasis is the least severe. It is endemic in over 70 countries. The yearly incidence is estimated at 1.5 million cases. Over 90% of the cases of CL occur in Afghanistan,

Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil, and Peru. The geographical distribution of CL is mainly determined by sand fly vectors (*Phlebotomus* sp and *Lutzomyia* sp). They live in dark, damp places and are relatively weak flyers, with a range of only 50 meters from their breeding site. They are most active in the evening and at night.¹

Leishmania researchers agree on the following:

- 1) In vertebrates, including human hosts, *Leishmania* species are obligate intracellular parasites of mononuclear phagocytes^{2,3};
- 2) There are 2 forms of the parasite: the amastigote form, which is intracellular and specifically found in the vertebrate hosts including humans, and the promastigote form, predominately found in the insect vector and not found in humans⁴; and
- 3) The demonstration of the amastigotes and not the promastigote form in lesions plays an important role in the diagnosis of CL.^{5,6}

Following a sand fly bite, some of the flagellates, once in circulation, enter the cells of the reticuloendothelial system, and there they transform into amastigote forms. The amastigote forms then multiply by binary fission within the macrophage until the host cell is packed with the parasites and ruptures, liberating the amastigotes into circulation. Then the free amastigotes invade fresh cells, thus repeating the cycle. In the process, some of the free amastigotes are drawn by the sand fly during its blood meal, thus completing the cycle.²

Pathology of the Disease

According to Hepburn, "over the following months, there is a gradual decrease in the number of amastigotes and macrophages, leaving a granulomatous infiltrate consisting of lymphocytes, epithelioid cells, and multinucleate giant cells.

At this stage it may be difficult or even impossible to detect organisms in H&E, or Giemsa-stained sections.” In the clinical features, he writes, “most patients have 1 or 2 lesions, usually on exposed sites, varying in size from 0.5 to 3 cm in diameter. There is, however, considerable variation: some lesions do not ulcerate, others develop sporotrichoid nodular lymphangitis. Most lesions heal over months or years, leaving an atrophic scar.”¹

An interesting question arises: why is healing not complete without a permanent scar?

Referring to Sharquie and colleagues, “the morphology of LD bodies (amastigotes) in smears assumed mainly spindle shape, other morphological forms like barrel, safety pin and umbrella-like were noticed, while the morphology in histopathological sections were rounded with a nucleus and kinetoplast. However, in some sections spindle shape form similar to smear morphology was detected. LD bodies were seen in histopathological sections in 30% of patients. Other histopathological features were mainly abundant with lymphocytes and plasma cells in the wet ulcerative lesions while in dry nodular types there was a tendency to form granuloma with less lymphocytes and scanty plasma cells.”⁷

This raises another very interesting question: where did the amastigotes and their macrophages disappear to causing the LD bodies to be seen in histopathological sections in only 30% of patients? And as long as the lesions continue months to a year with no amastigote presence (which means the disappearance of the causing agent of the disease), is it possible that any kind of amastigote transformation has happened and a new form was missed by microscopic smear exam?

Methods of Transmission

The predominant mode of transmission is the bite of a sand fly; however, there are also uncommon modes of transmission through congenital transmission, blood transfusion, and, rarely, through inoculation of cultures.² In some regions, natives inoculate their children in a site normally not visible to protect the child from developing disfiguring scars later in life.⁸

With other modes of transmission aside from the bite of a sand fly raises doubts about the absoluteness of the sand fly vector for the parasite to complete its life cycle, and thus assuming the possibility of the promastigote form to be present in the extracellular fluid of the infected vertebrate.

Materials and Methods

Since 1999, we have received 42 patients clinically diagnosed with CL referred to us from consultant dermatologists for confirmatory laboratory diagnosis. Our method of identification was parasite diagnosis by microscopic examination of the skin lesion, which remains the “gold standard” with its usual limitations.

Samples

Patients referred to the laboratory had samples collected from the lesion area of the skin and microslides were prepared. Two slides were prepared from each lesion. In cases where there were more than 2 exposed areas on the same patient, we chose the 2 more edematous lesions and took 2 slides from each, stained with Wright stain.

All the smears for each case were reserved and numbered as such: case one (1c), case one slide one (1c1), case 2 (2c), and so forth. Comparison was made among the microscopic features of the parasite in the different slides as follows:

- 1) A study was done for counting the amastigote appearance in the intracellular or extracellular location in all cases.
- 2) Another study was done to identify and compare the manifestations of the different shapes of the amastigotes seen in the extracellular fluid among the cases referred.
- 3) Then a study was done for locating the presence of any type of suspicious organism appearing different than any of the blood or the human dermal tissue components.
- 4) A morphologic comparison was made on the discovered flagellates among the different smears taken from the different cases referred to us.
- 5) Another morphologic comparison was made between the presumed fiber-forming promastigote form found, and fibroblasts in skin biopsy specimens stained with H&E stain to rule out the difference.

Six hundred documentary microscopic photos were taken for the presumed parasite forms discovered for confirmation, documentation, and comparison with other reference photos for the parasite in both its amastigote and promastigote forms.

Results

Table 1 illustrates the intracellular or extracellular presence of the amastigote, and it reveals that out of 42 cases referred to the laboratory, microscopically, only 1 case (2.4%) showed the presence of the amastigote intracellularly alone but not in the extracellular area; 2 cases (4.75%) showed the presence of the amastigote in the extracellular but not in the intracellular location; and in 20 cases (48%), we microscopically observed the amastigote presence in the intracellular or extracellular area. Out of the 20 cases in which the amastigote was recovered, we were able to identify the amastigote in 17 cases (85%) in the intracellular and extracellular locations simultaneously. Additionally, in all 42 cases referred, the amastigote form alone—without no promastigote associated—was microscopically recovered in 10 cases (24%).

Table 2 shows other forms found in the microscopic slides of the 42 patients and reveals that out of 42 cases referred to the laboratory, microscopically, the promastigote-like form was found in 32 (76%) of the cases referred, and the amastigote form was recovered associated with the promastigote-like in 10

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Table 1_Presence of the Amastigote Form

| Amastigote Form | Intracellular Alone | Extracellular Alone | Only Amastigote Present With No Promastigote Form | Amastigote Present Inside or Outside the Macrophage |
|-----------------|---------------------|---------------------|---|---|
| No. of cases | 1/42 | 2/42 | 10/42 | 20/42 |
| Percentage | 2.38 | 4.75 | 24 | 48 |

Table 2 Presence of Promastigote-Like Forms

| Promastigote-Like Forms | Seen in the Smear | Associated With the Amastigote Forms | Present Alone in the Smear (With no Amastigote Forms) |
|-------------------------|-------------------|--------------------------------------|---|
| No. of cases found | 32/42 | 10/42 | 22/42 |
| Percentage | 76 | 24 | 52 |

cases (24%) of the total. The promastigote form was recovered alone without the amastigote combination in 22 cases (52%).

Discussion

Table 1 indicates that the amastigote forms are almost available in both intracellular and extracellular fluid simultaneously every time the amastigote form is found in the smear. That presence in the extracellular fluid demonstrates the amastigote's ability to survive in the extracellular environment not necessarily the same as inside the macrophage, but at least as a bridge for transformation into the promastigote-like form. This leads to the conclusion that, in humans, the amastigote form (contrary to the previous studies) is not an obligatory intracellular parasite that cannot survive in the extracellular environment as long as we see it in both locations at almost the same rate.

From **Table 2** we concluded that the images (**Images 1–12**) have proven the presence of other forms apart from the amastigote form in the extracellular fluid. We called these forms “promastigote-like” as a general term, and, according to their developmental stage, and relying on the photos taken from the slides, we classified them in the following order:

1) Once outside the macrophage, the amastigote form becomes like an ova containing the promastigote embryo. The nucleus is usually centralized, the surrounding cytoplasm has a spindle-like shape, and the chromatin is smooth (**Images 1, 2, and 5**).

2) Inside the amastigote, the nucleus starts to take a polarized position, and the cytoplasm appears on one side of the amastigote. At the same time, the chromatin shows more condensation (**Images 1 and 2**).

3) This developing embryo becomes larger in size and more condensed, assuming a candle flame shape while the cytoplasm disappears (**Images 1 and 8**).

4) The embryo continues to grow with a small tail protruding outside (**Images 1, 7 and 8**).

5) The tail continues growing, taking a flagella shape, together with the whole organism increasing in size from 2 to 3 microns to approximately 4 to 6 microns, and starting to assume a small promastigote shape (**Images 7 and 8**).

6) In this stage, the promastigote becomes fully mature with dimensions approximately 8 to 12 microns in diameter (**Images 3–6 and 8**).

Sharquie and colleagues wrote, “the morphology of LD bodies (amastigotes) in smears was mainly spindle in shape, other morphological forms like barrel, safety pin and umbrella like were noticed.”⁷ In fact, they described the transforming stages of the amastigote to promastigote-like but were not able to identify the transformation significantly.

7) Transformation into fiber-forming promastigote stage. In this stage the promastigote-like form starts transforming into fiber production where the flagella becomes more condensed, thickened, and enlarged to approximately 40 microns or more

in length (**Image 10**). We may also see the fiber formation not only from the flagella position but also from the opposite side (**Image 12**), or occasionally at different poles (**Image 11**). This fiber structure formation in the derma may react like a foreign graft and explain the inflammatory fiber granulomatous immune reaction associated with the lymphocytes, mononuclear cells, and variable number of plasma cells (graft rejection-like reaction).

Once again, Sharquie and colleagues described that “other histopathological features were mainly abundant of lymphocytes and plasma cells in the wet ulcerative lesions, while in dry nodular types there was a tendency to form granuloma with less lymphocytes and scanty plasma cells.”⁷ They were able to describe the histopathological condition of the disease without connecting it with the etiology, which is the presence of the promastigote in the wet lesion and the presence of fibers and fiber-forming parasites in the dry nodular type of lesions. Our findings indicated we could see a mixture of both histopathological features mentioned at different rates, in different smears according to a certain stage of the disease case.

8) The last phase: conversion to fibers. Here the fibers are loaded, elongated, and the parasite nucleus becomes thinner, smaller, thready, and more condensed in the middle, along with the fiber continuing from both sides (**Image 9–12**). It is as if the parasite embalms itself. At this point, few lymphocytes and plasma cells are seen and, clinically, the lesion is dry and close to healing and forming a permanent scar.

The formation of the post-healing lifetime scar is justified by the production of these types of pseudo-fibers in the lesion by the parasite itself and not the human fibroblasts. Apparently, those remaining foreign fibers formed and inserted in the last stage of the lesion healing will stimulate and become a focal area of permanent immune inflammatory reaction, and this explains in part the incomplete healing of the skin in the area with resulting permanent scar formation.

Some researchers went through this phenomena. Hepburn wrote, “there is, however, considerable variation: some lesions do not ulcerate, others develop sporotrichoid nodular lymphangitis. Most lesions heal over months or years, leaving an atrophic scar,”¹ but without detecting the reason of this lifetime scar. \square

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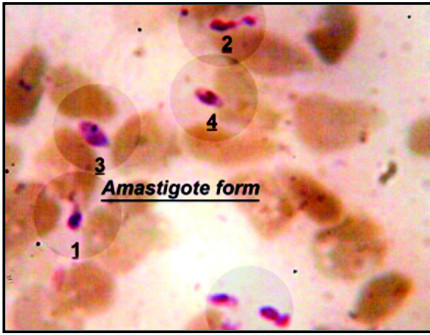


Image 1

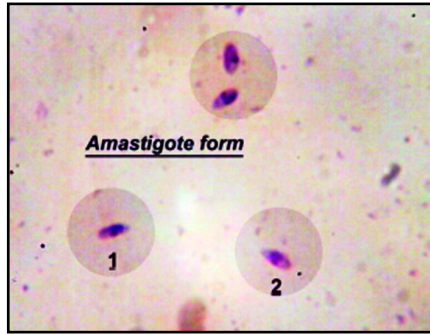


Image 2

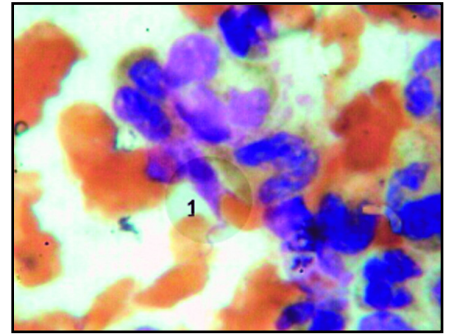


Image 3



Image 4

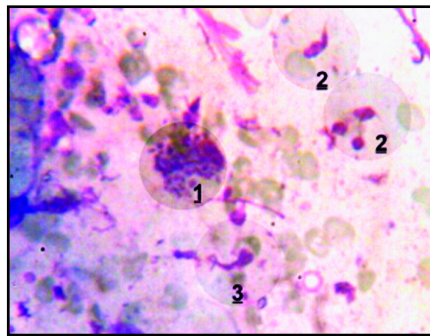


Image 5

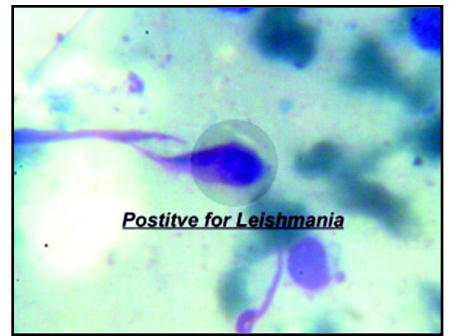


Image 6

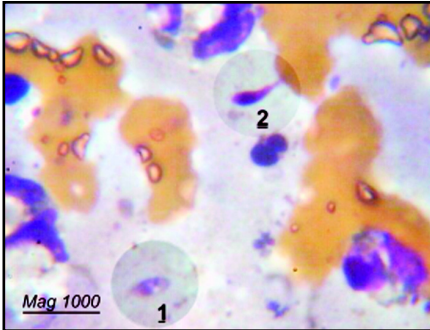


Image 7

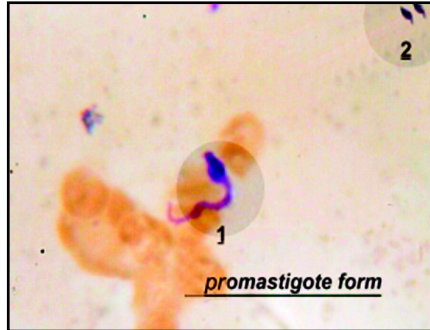


Image 8

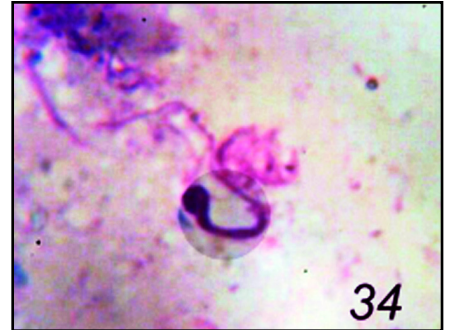


Image 9



Image 10

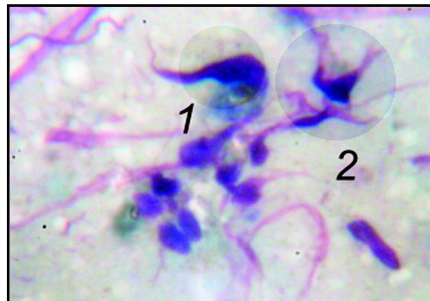


Image 11

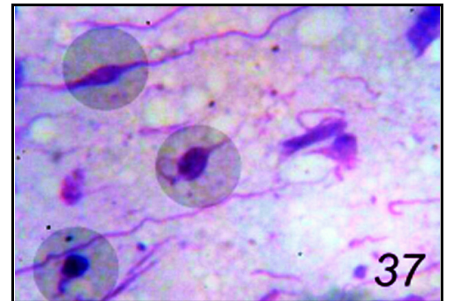


Image 12

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