Pharmacogn J. 2023; 15(5): 905-909
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Research Article

Generation of Multinucleated Giant Cells due to *Leishmania* (V.) *braziliensis* Infection

Jesús Rojas-Jaimes[1,2,*], Luis Castañeda Pelaez[1]

ABSTRACT

Background: Multinucleated giant cells were first described by Langhans(MGCs), these MGCs have a role in innate immunity that includes extracellular matrix remodeling associated with granuloma formation; another function of them is to participate in the removal of cellular debris in apoptosis during certain infections. **Objective:** Generate multinucleated giant cells due to *Leishmania* (*V.*) *braziliensis* infection. **Methods:** For infection macrophage/parasite ratio of 1 to 10 using the RAW 264.7-line (5 x10^5/mL) and *Leishmania* (*V.*) *braziliensis* metacyclic promastigotes (50 x10^5/mL) was a strain maintained in culture Schneider liquid medium, supplemented with 20% fetal bovine serum and 10 000 U/10 mg/mL penicillin streptomycin at pH 7 and 25 °C in the laboratory. Incubation was continued for 4 days, and microscopic observation at 1000X was performed at 24 hours and 96 hours, respectively. **Results:** In our study an MGC conversion was observed at 96 hours (50% ± 28.2). **Conclusions:** *In vitro* MGC model could be used to study the physiopathology of MGC generation by *Leishmania* (*V.*) *braziliensis* infection.
Key words: *Leishmania* (*V.*) *braziliensis*, Multinucleated Giant Cells, Infection, Immunity, Macrophage.

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History

- Submission Date: 20-07-2023;
- Review completed: 02-09-2023;
- Accepted Date: 06-09-2023.

DOI : 10.5530/pj.2023.15.172

Article Available online

http://www.phcogj.com/v15/i5

INTRODUCTION

A Leishmaniasis is a disease caused by the parasite *Leishmania* and is transmitted by a dipteran of the genus *Lutzomyia* or *Phlebotomus.* Leishmaniasis is a growing public health concern and is considered endemic in 88 countries. Leishmaniasis is one of the most neglected tropical diseases whose etiological agent is intramacrophage protozoa, which maintains its life cycle through transmission between the sandfly and a mammalian host. The clinical manifestations of this zoonosis will depend on the complex interactions that they are the result of invasiveness, tropism, pathogenicity, and the patient's immune response that is genetically determined.[1-4] There are three varieties of leishmaniasis: cutaneous leishmaniasis, mucosal leishmaniasis, and visceral leishmaniasis. According to previous research, these varieties are associated with pathological clinical manifestations.[1,2]

Leishmania (*V.*) *braziliensis* produces the mucocutaneous leishmaniasis known as espundia, which represents a dreaded complication that causes disfigurement in the patient and is typical for the most part in South America. While in the old world *Leishmania tropica* or other *Leishmania spp*, their mucosal involvement is occasionally caused by the contiguous spread of skin lesions.[1-4]

The complexity of leishmaniasis is due to the union of the countless feasible combinations of different syndromes of the disease, species and geographical areas where the infection is obtained, consequently, each combination varies according to its clinical manifestation, ease of diagnosis, natural history and response to treatment.[5]

In leishmaniasis, human is the accidental host and canids and rodents are natural hosts, being this disease endemic to areas of the tropics, subtropics and southern Europe, in environments ranging from tropical forests in the Americas to deserts in Asia. western, and from rural to peri-urban areas.[5,6]

One of the species of particular concern is *Leishmania* (*V.*) *braziliensis.* There is evidence that *Leishmania* (*V.*) *braziliensis* can lead to mucosal leishmaniasis in 5% of patients, affecting the upper respiratory tract and destruction of adjacent tissues.[5,6] These studies suggest that patients are at high risk of coinfections and may suffer further health complications.

Multinucleated giant cells were first described by Langhans.[7] The formation of multinucleated cells in healthy people can be found in bone tissue.[8] However, multinucleated giant cells (MGCs) in non-skeletal tissues are the result of chronic inflammation due to the presence of foreign material that is poorly digestible or persistent pathogens that are not eliminated for various reasons. These MGCs have a role in innate immunity that includes extracellular matrix remodeling associated with granuloma formation; another function of them is to participate in the elimination of cellular debris in apoptosis during certain infections.[9,10]

Previous research findings into MGCs derived from the fusion of a macrophage monocyte cell line may be involved in the formation of granulomas with lymphoplasmacytic halo and fibrosis.[11-16] Because of this, chemokines such as CXCL8 contribute to inflammatory cells in the site of infection and cytokines such as IFN-ɣ, IL-4 e IL-13.[11-16]

Macrophages play several important roles in host defense as they have the ability to ingest and destroy microorganisms, remove dead tissue and initiate the process of tissue repair, and produce cytokines that induce and regulate inflammation. These cells that are part of innate immunity and that are the link with acquired immunity present recognition receptors including those of the toll type (TLR, toll-like receptor) and those of the NOD type (NLR,

Cite this article: Rojas-Jaimes J, Pelaez LC. Generation of Multinucleated Giant Cells due to *Leishmania* (V.) *braziliensis* Infection. Pharmacogn J. 2023;15(5): 905-909.

NOD-like receptor) are a large family of innate receptors that detect DAMP and PAMP in the cytosol of cells and initiate signals that promote inflammation. These receptors presented by macrophages recognize products of microorganisms and damaged cells and activate macrophages.[14-16]

Macrophages have the ability to activate by two different pathways, one is the classical activation pathway and the other is the alternative activation pathway, this allows them to serve different functions. Classic activation of the macrophage, also called macrophage type 1 (M1) and activation by the alternative pathway also called macrophage type 2 (M2).[14-16]

M1 macrophages engulf and kill microorganisms, kill tumor cells, and present antigens to T cells to trigger a specific adaptive immune response. In addition, they secrete anti-tumor mediators such as IL-1β, IL-6, IL-12, IL-18, IL-23, tumor necrosis factor alpha (TNF-α) and express high levels of the major complex of type I and II histocompatibility. While macrophages with the M2 phenotype, on the other hand, are stimulated by other types of interleukins such as IL-4, IL-13, IL-21 and IL-33, more of an anti-inflammatory nature, by immune complexes and by glucocorticoids, and produce interleukins such as IL-4, IL-5, IL-10 and IL-13 and other components involved in humoral immunity and healing.[14-16]

The cell line RAW264.7 cells, are originally derived from tumors induced with Abelson leukemia virus. In most tumors, macrophages are considered to be of the M2 type, due in part to the absence of M1 signals, providing a microenvironment that favors tumor growth. In addition, M2 TAMs (Tumor Associated Macrophages) secrete many cytokines, chemokines, proteases and growth factors that promote tumor angiogenesis, thus favoring tumor spread and metastasis, thanks to nutrients and oxygen supplied to the tumor by the new blood vessels. These M2 macrophages are the perfect aid in this process, especially the wound healing subset, as they are primed to remodel tissue and produce Vascular Endothelial Growth Factor (VEGF).[16]

Evidence suggest that MGCs have been observed in several bacterial infections. A number of studies have shown that *Mycobacterium tuberculosis*, *Entamoeba invadens* and HIV were found in MGCs.[17-20] These studies clearly indicate that this is a cellular response to infection by various pathogens

The purpose of the MGCs is to remodeling the matrix of granuloma, remove external particles and waste generated by apoptosis.[21-23] Among the cytokines involved in the generation of MGCs are the macrophage colony-stimulating factor (M-CSF), the nuclear factor-activated receptor, TNF α, lipopolysaccharides (LPS) y and the role of NADPHD oxidase.[21-23]

Morphologically multinucleated giant cells are generally classified into Langhans giant cells and foreign body giant cells (FBGCs). Langhans giant cells are characterized by having a relatively small number of nuclei, less than 20, which are arranged in a circular peripheral fashion within the giant cell. FBGCs usually present a large number of nuclei, greater than 20 and irregularly located throughout the giant cell.[9,13]

Langhans giant cells are regularly seen in granulomas with epithelioid-like macrophages and are associated with indigestible particles of organisms surrounded by a necklace of mononuclear leukocytes that are essentially lymphocytes. FBGCs are observed at the tissue-material interface of medical devices implanted in soft and hard tissues.[17]

Additionally, FBGCs have been implicated in biodegradation events of polymeric medical devices. FBGC and macrophages shape the foreign body reaction at the tissue-device interface and are surface area dependent. Tissues that are used as vascular grafts show high densities of FBGC, whereas flat surfaces such as those found in breast implants exhibit only a layer of one or two macrophage cells and FBGC at the tissue-material interface.[9,13]

There are apparent differences in the mechanisms of *Leishmania* clearance elaborated by human macrophages, such is the case that reactive nitrogen species play a minor role in the control of leishmaniasis, studies have indicated that infection with amastigotes results in limited production. of superoxide, one possible explanation for why superoxide might play a limited role in the control of leishmaniasis, is that infection with the amastigotes form of the parasite results in limited superoxide production.[23]

The aim of this study was to determine the formation of multinucleated giant cells due to *in vitro* infection by *Leishmania* (*V.*) *braziliensis.*

MATERIALS AND METHODS

Cell line

Mouse macrophage commercial cell line RAW264.7 cells, originally derived from tumors induced with Abelson leukemia virus, were obtained from ATCC (ATCC TIB-71) through a purchase. Cells were maintained at 37˚C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM: Sigma-Aldrich, Japan) containing 10% heat-inactivated fetal bovine serum (HI-FBS) (Thermo Fisher Scientific, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo®).

Growth calibration curve of mouse macrophage commercial cell line RAW264.7

The optimal day of cell growth was determined on a logarithmic scale related to its optimal metabolic state, performing a daily count in a Neubauer chamber using the formula: Cells/mL =[(# cells counted) (Dilution factor) (1/Volume factor)]x 1000 . The reading was carried out until the next day, when the growth peak was reached on the cellular logarithmic scale.

Growth calibration curve of *Leishmania (V.) braziliensis*

The optimal day of parasite growth was determined on a logarithmic scale related to its optimal metabolic state, performing a daily count in a Neubauer chamber using the formula: Parasites/mL = [(# parasites counted) (Dilution factor) (1/Volume factor)]x 1000. The reading was carried out until the next day, when the growth peak was reached on the parasite logarithmic scale.

Cell infection

For infection 24-well sterile polystyrene plates were used and each well contained a macrophage/parasite ratio of 1 to 10 using the RAW 264.7-line (5 x10^5/mL) and *Leishmania* (*V.*) *braziliensis* metacyclic promastigotes (50 x10^5/mL) was a strain maintained in culture Schneider liquid medium, supplemented with 20% fetal bovine serum and 10 000 U/10 mg/mL penicillin streptomycin at pH 7 and 25 °C in the laboratory. Incubation was continued for 4 days, and microscopic observation at 1000X was performed at 24 hours and 96 hours, respectively, using slides with cavities which were fixed at room temperature in a laminar flow cabinet for 5 minutes and, then, stained with Giemsa stain. The reading at 24 hours and 96 hours was performed in triplicate and 100 fields were observed for each reading.

Statistical analysis

From the triplicates, the percentages of infected cells at 24 and 96 hours were calculated. Similarly, MGCs percentages were calculated at the evaluated hours with standard deviation.

RESULTS

Results are shown in table 1 and figures 1 to 3.

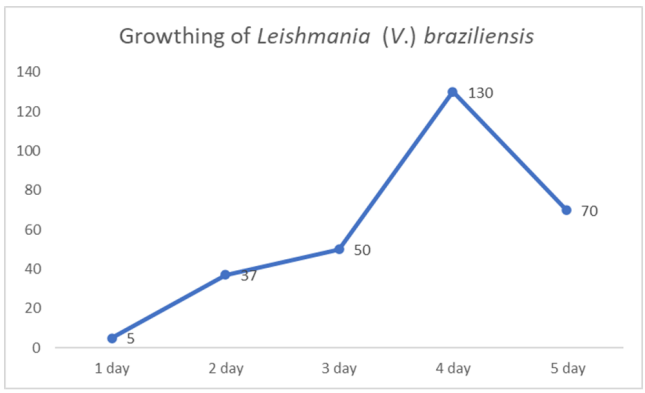

Figure 1: Growth per day of *Leishmania* (*V.*) *braziliensis*
*The optimal growth of metacyclic promastigotes for the infection test was given in logarithmic phase x 10 ^{5}/mL on the third day.

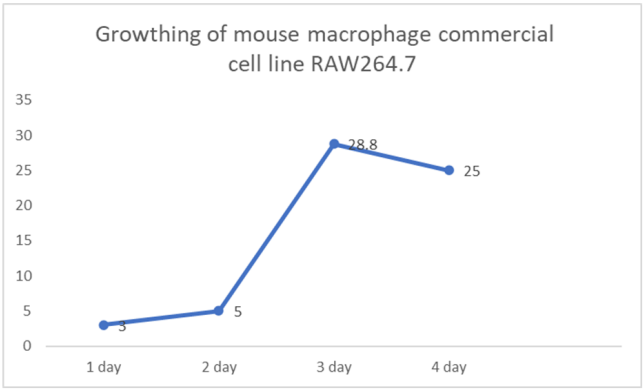

Figure 2: Growth per day of mouse macrophage commercial cell line RAW264.7 cells
*The optimal growth of mouse macrophage commercial cell line RAW264.7 cells for the infection test was given in logarithmic phase x 10 ^{5}/mL on the second day.

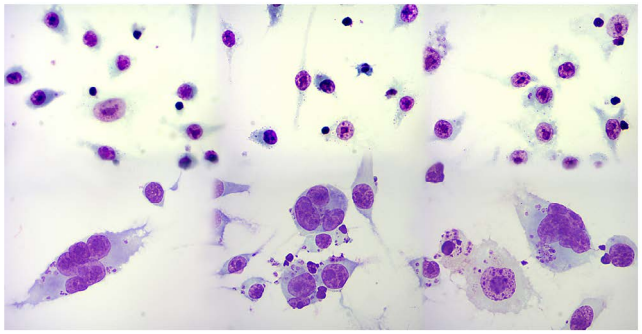

Figure 3: On top, RAW264.7 macrophages infected with *Leishmania* (*V.*) *braziliensis* at 24 hours showing mononuclear cells in 3 fields (100X). On the bottom, RAW264.7 macrophages infected with *Leishmania* (*V.*) *braziliensis* at 96 hours showing multinucleated giant cells in 3 fields (1000X).

Table 1: Percentage of infection and MGCs generated by *Leishmania* (V.) *braziliensis*.

	24 hours	96 hours
Percentage of infection	25% ± 3	83% ± 14.7
Percentage of MGCs	0%	50% ± 28.2

The table illustrates that the percentages of infection increased as time elapsed. In the case of the generation of MGCs, they were only generated at 96 hours with a value of 50% ± 28.2.

DISCUSSION

A previous study has shown how RAW 264.7 MGCs infected with *Leishmania donovani* perform hemophagocytosis of red blood cells and stimulate *Leishmania* survival through the glycoprotein SIRPα (a molecule involved in "don't eat me" cell recognition), in which a decrease of SIRPα expression was found in infected cells performing hemophagocytosis. These results coincide with those of Hong *et al.* where bone marrow-derived macrophages (BMDM) infected with *L. donovani* were co-cultured with erythrocytes, this led to the cells having a higher rate of hemophagocytosis compared to uninfected bone marrow-derived macrophages (BMDM). Furthermore, BMDMs infected with *L. donovani* and stimulated with GM-CSF or IFN-γ showed higher rates of hemophagocytosis,[24,25] inferring that this mechanism is manipulated by the parasite as a survival mechanism within the MGCs. In our study it was observed that 24 hours after infection there were no MGCs with an average infection rate of 25%±3 (Figure 3), and, in the case of observation at 96 hours, an infection rate of 83%±14.7 was determined with 50%± 28.2 of MGCs (Figure 3), in optimal growth scales of the macrophage and the parasite on the fourth and fifth day respectively. (Figures 1 and 2)

One study found that at 72 hours infection of macrophages by *donovani* y *L. major* generated MGCs of MGCs 39.2% ± 4.2% and 36.2% ± 6.9% respectively.[25] However, this study found that infection was 83%±14.7 and MGCs generated by infection of *L.* (*V.*) *braziliensis* at 96 hours was 50%±28.2. This result differ from a previous study were the percentage in our study was higher.[25]

In vivo, the presence of MGCs infected by *Leishmania* may serve as an indicator of relapse and hemophagocytosis processes, as previously observed in studies.[10,24] This finding is consistent with a previous study where 71.43% of patients with mucosal leishmaniasis exhibited MGCs with granuloma formation and chronic inflammatory reactions.[26] In addition to this, it is noteworthy that molecules involved in the formation of MGCs include IL-4, el IL-13, IFNγ and ICAM -1 respectively.[27-29]

In one study they found that BMDMs treated with cytokines including GM-CSF, IL-4 and IFNγ promoted MGC formation in *Leishmania donovani* infection whereas BMDMs not treated with cytokines or only stimulated either by IL-4 or by INFγ did not show apparent MGC formation. In addition, cytokines including GM-CSF, IL-4, and IFNγ are inducers of multinucleation and the formation of phenotypically distinct MGCs. These results coincide with those of Fais *et al.* and it would also be the case of our research that after 96 hours the presence of MGC was observed, this could be due to stimulation with cytokines including GM-CSF, IL-4 and IFNγ that promoted the formation of MGC.[25,29]

As mentioned in the literature, the formation of granulomas with MGCs has been described in animals such as horses.[30] This suggest that the generation of MGCs is a reaction to limit the dissemination of etiological agents in mammals. However, recent reports showed that MGCs are implicated in pathological processes such as sarcoidosis and rheumatoid arthritis.[31,32]

Prior studies have shown that MGCs were found in cases of visceral and cutaneous leishmaniasis[33,34] as well as *In Vivo* infections in murine models such as the BALB/c.[35,36] In this study, the formation of MGCs was induced *In Vitro* by *L.* (*V.*) *braziliensis.*

In an investigation, it was reported that histological studies of the spleens of mice infected with promastigotes of *L. donovani* showed hemophagocytosis in macrophages with high infection with amastigotes. In addition to being heavily infected, the multinucleated giant cell (MGC) phenotype was prominent in those hemophagocytes. Multinucleated macrophages represented 15.0 ± 6.2% of the total splenic macrophages,[35] in our experimental work they were carried out

in vitro with the cells of the commercial mouse macrophage cell line RAW264.7, originally derived from induced tumors. with Abelson's leukemia virus, ATCC (ATCC TIB-71), a percentage of 50% ± 28.2 of MGC generated by *Leishmania (V.) braziliensis* was obtained.

The multinuclear phenotype was highest in hemophagocytes where 60.4 ± 5.8% of mouse splenic hemophagocytes were multinucleated.[35] Some multinucleated macrophages were also found in both liver and bone marrow tissues of mice, the proportion in those tissues being lower than that in the spleen,[35] this would be because the spleen is a larger lymphoid organ to remove. particulate matter in the blood and concentrates hardening and microorganisms.

In addition, the spleen presents the white pulp is the main lymphatic tissue of the spleen. It is the accumulation of lymphocytes around an arterial vessel. This aggregation of lymphocytes constitutes the lymphatic tissue known as the periarterial lymphatic sheath and is the first to react if microorganisms reach the spleen through the bloodstream. The red pulp is made up of splenic venous sinuses and cords (of Billroth), linings of splenic macrophages around the sinuses. The central artery continues from the white pulp and enters the red pulp in the form of capillaries.[37] In visceral leishmaniasis, the spleen is infected by *Leishmania*, therefore it is possible to expect that MGC can also be found in this organ in *In Vivo* infections, so it is necessary to determine the percentage and pathophysiology of this type of cells.

A possible explanation for this might be that the formation of MGCs is due to the interaction and recognition between macrophages and cellular structures. It has been suggested that MGCs did not form when latex microspheres were administered to macrophages, indicating that phagocytic capacity and MGC formation are linked to distinct pathways.[25]

CONCLUSION

In our study an MGC conversion was observed at 96 hours (50% ± 28.2) which would be a reaction due to cytokine overstimulation with IFNγ trying to control parasitaemia and an overactivation of macrophages. In addition, *in vitro* MGC model could be used to study the physiopathology of MGC generation by *Leishmania* (*V.*) *braziliensis* infection.

FUNDING

This work had no source of funding.

AUTHOR CONTRIBUTIONS

J.R-J made a substancial contribution in the conception, design and data collection; L.C-P contributed in data analysis, draft the work and revisión. Both authors approved the final versión of the article.

INFORMED CONSENT STATEMENT

Not applicable

DATA AVAILABILITY STATEMENT

Not applicable

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Cite this article: Rojas-Jaimes J, Pelaez LC. Generation of Multinucleated Giant Cells due to *Leishmania* (V.) *braziliensis* Infection. Pharmacogn J. 2023;15(5): 905-909.