Immunohistochemical characterization of the cellular infiltrate in Jorge Lobo’s disease

Fátima Regina Vilani-Moreno¹, Andréa Faria Fernandes Belone¹, Cleverson Teixeira Soares² & Diltor Vladimir Araújo Opromolla³

¹Equipe Técnica de Biologia, ²Equipe Técnica de Patologia and ³Divisão de Pesquisa e Ensino, Instituto Lauro de Souza Lima, Bauru-SP, Brazil

Summary

Few studies have been conducted to evaluate the cellular composition of the granulomatous lesions induced by Lacazia loboi. Thus, the objective of the present study was to characterize the mononuclear cell population present in cutaneous lesions obtained from 15 patients with Jorge Lobo’s disease. Histological sections were stained with hematoxylin-eosin and methenamine silver and the following mononuclear cells were identified by immunohistochemistry: T lymphocytes (CD3⁺), helper T lymphocytes (CD4⁺), cytotoxic T lymphocytes (CD8⁺), B lymphocytes (CD20⁺), plasma cells (CD79⁺), natural killer cells (CD57⁺) and histiocytes (CD68⁺). This study showed that the inflammatory infiltrate mainly consists of histiocytes and multinucleated giant cells, in addition to the presence of a large number of fungal cells. The identified inflammatory cells showed the following frequency: CD68⁺ histiocytes > CD3⁺ T lymphocytes > CD4⁺ T > CD8⁺ T lymphocytes > CD57⁺ natural killer cells > CD79⁺ plasma cells > CD20⁺ B lymphocytes. Based on the findings of a large number of fungal cells in the infected tissues and the disorganized cell arrangement in the granuloma, we hypothesize that patients with Jorge Lobo’s disease present immunoregulatory disturbances, which are likely to be specific and perhaps responsible for the lack of containment of the pathogen.

Key words

Jorge Lobo’s disease, Lacazia loboi, Immunohistochemistry, Mononuclear cells

Identificación imunohistoquímica del infiltrado inflamatorio de la enfermedad de Jorge Lobo

Resumen

Considerando la escasez de estudios sobre la composición celular del granuloma inducido por el Lacazia loboi y el pequeño número de pacientes evaluados, hemos estudiado la población de células mononucleares presentes en las lesiones cutáneas de 15 pacientes portadores de la enfermedad de Jorge Lobo. Se tiñeron los cortes histológicos con hematoxilina-eosina, plata metenamina y con el método imunohistoquímico se identificaron las siguientes células mononucleares: linfocitos T (CD3⁺), linfocitos T auxiliares (CD4⁺), linfocitos T citotóxicos (CD8⁺), linfocitos B (CD20⁺), plasmócitos (CD79⁺), células NK (CD57⁺) e histiocitos (CD68⁺). Los resultados obtenidos revelaron que el infiltrado inflamatorio estaba compuesto predominantemente por histiociotos y células gigantes multinucleadas, además de un gran número de hongos. La frecuencia de células encontradas fue la siguiente: histiociotos CD68⁺ > linfocitos T CD3⁺ > linfocitos T CD4⁺ > linfocitos T CD8⁺ > células NK CD57⁺ > plasmócitos CD79⁺ > linfocitos B CD20⁺. Así, considerando los resultados obtenidos, en los que observamos una gran cantidad de hongos en las lesiones y una disposición desorganizada de las células en el granuloma, podemos sugerir que los pacientes con la enfermedad de Jorge Lobo presentan alteraciones imunorregulatorias, probablemente específicas, responsables de la no contención del patógeno.

Palabras clave

Enfermedad de Jorge Lobo, Lacazia loboi, Imunohistoquímica, Células mononucleares
Jorge Lobo’s disease is a chronic, cutaneous-subcutaneous mycosis caused by *Lacazia loboi*, a fungus phylogenetically closely related to the dimorphic pathogen *Paracoccidioides brasiliensis* [5,11,23]. The mycosis is predominantly found in Brazil, but cases have been reported, in decreasing order, in Colombia, Surinam, French Guyana, Venezuela, Panama, Costa Rica, Peru, Ecuador, Bolivia, Mexico, and Europe [21]. According to Opromolla et al. [18], the estimated number of cases of the disease is 458, 295 of them occurring in Brazil, all of them in the Amazon region.

Jorge Lobo’s disease has been described in different human populations, and there is no evidence of a greater susceptibility to the infection on a given ethnic group [8]. Clinically, the mycosis is characterized by cutaneous lesions with a keloid-like, verruciform, infiltrative, ulcerative or gummy aspect, with the same patient sometimes presenting more than one type of lesion [22]. Opromolla et al. [18] have classified these lesions according to their localization and distribution into localized lesions – restricted to a single area, multifocal lesions involving various distinct anatomical regions.

Histopathologically, Jorge Lobo’s disease is characterized by an intense and diffuse histiocytic reaction consisting of large numbers of foreign body-type and Langhans giant cells and numerous fungi mainly inside these cells; lymphocytes, plasma cells and neutrophils are present in smaller numbers [8,9,17]. The occurrence of fibrosis, sometimes exacerbated, is a frequent finding in this disease; however, in contrast to other mycoses such as paracoccidioidomycosis and chromomycosis, no acute inflammation or necrosis is observed [8,9]. Several authors have reported the destruction of cutaneous appendages and of nerve endings in the skin as a result of the invasion of the dermis by the histiocytic infiltrate [8,9]. However, Opromolla et al. [17], in a histopathological study of the lesions of 40 patients with the disease, did not observe involvement of vessels, nerves or appendages. Epidermal alterations have also been reported in Jorge Lobo’s disease. Wiersema [29] described the simultaneous presence of focal atrophic and hypertrophic and normal areas in the same epidermal lesion; in the case of an altered epidermis, hyperkeratosis and, sometimes, parakeratosis are usually observed. These findings were also reported by Baruzzi et al. [1] who analyzed 15 cases of the mycosis, and by Opromolla et al. [17] in 65% of 40 cases studied.

Little is known about the characterization of the inflammatory cellular population in cutaneous lesions of patients with the mycosis. Only two papers in which four patients were microscopically evaluated are available. For instance, Esterre et al. [3] evaluated the lesions of three patients with the mycosis and observed a predominance of macrophages, few T lymphocytes usually of the CD4+ phenotype, and the absence of B lymphocytes. Ribeiro-Rodrigues et al. [20] evaluated the lesion of a unique patient and found an absolute predominance of histiocytes (CD68*) and the absence of T (CD3*) and B (CD20*) lymphocytes.

The objective of the present study was to characterize the mononuclear cell population present in the cutaneous lesions of patients with Jorge Lobo’s disease to better understand the host-parasite relationship of this mycosis.

**Patients and methods**

**Patients.** Fifteen patients with Jorge Lobo’s disease originating from the State of Acre, Brazil, were evaluated by the clinical staff of the Lauro de Souza Lima Institute, Bauru, Brazil, and the disease was confirmed by anatomopathological examination. All patients were males with a mean age of 51 years, with no history of drug management. Nine of these patients showed single localized lesions and six had multicentric lesions.

**Cutaneous lesion sample.** A skin fragment of the lesion was obtained from each patient by biopsy or by complete surgical removal of the lesion as indicated. The specimen was fixed in 10% formalin for 24 h and processed by routine procedures for embedding in paraffin. Histological sections (4 µm) were stained with hematoxylin-eosin (HE) and Gomori’s methenamine silver [4]. The remaining material was used for immunohistochemical analysis.

**Identification of mononuclear cells in the cutaneous lesions.** The cell population of the inflammatory infiltrate was characterized by immunohistochemistry using immunoperoxidase labeling (Dako EnVision System, Dako Corporation, USA). Serial 4 µm thick sections were mounted on silane slides (Dako) and submitted to fixation, deparaffinization and hydration. The slides were then treated with 3% hydrogen peroxide in methanol (Merck, Germany) for 30 min to block endogenous peroxidases, and subsequently incubated with 10 mM citrate buffer, pH 6.0, and heated in a microwave oven to 95 °C during two cycles of approximately 5 min each for antigen recovery of the CD3, CD8, CD20, CD68 and CD79 markers (the CD57 marker does not require antigen recovery). After cooling, the slides were incubated with the primary antibodies diluted in bovine serum albumin (Inlab, Brazil) for 1 h at room temperature. The samples were treated with the Dako EnVision System for 30 min, followed by incubation with 1 mg/ml DAB solution (3,3’ tetrahydrochloride diaminobenzidine; Sigma, USA) in phosphate-buffered saline (PBS) pH 7.4, plus 1% hydrogen peroxide (Merck) for 5 min. The slides were counterstained with Harris’s hematoxylin and mounted in Permount resin.

The slides were washed with PBS between each reaction step. Histological sections of the tonsils labeled with each of the markers studied served as positive control, and samples in which the primary antibody was omitted were used as negative control. Positive labeling was identified by a brown staining around the cell membrane or by a brown color of the cytoplasm according to the marker employed. Table 1 lists the antibodies and dilutions used in the present study.

**Identification of helper T lymphocytes in the lesions.** CD4+ T lymphocytes were identified by immunohistochemistry using the Dako EnVision Doublestain System (K1395). Briefly, slides were submitted to fixation, deparaffinization, hydration and blockage of endogenous peroxidases. CD3 and CD8 marker antigens were recovered by incubation with 10 mM citrate buffer, pH 6.0, in a water bath at 95 °C for 30 min. After cooling, the slides were incubated with the primary anti-cytotoxic T lymphocyte (CD8) antibody for 30 min at room temperature, followed by incubation with the EnVision System (peroxidase-labeled polymer conjugated with goat anti-mouse and rabbit IgG polyclonal antibody) for 30 min at room temperature. After washing with PBS, the slides were incubated with DAB plus hydrogen peroxide, and then treated...
with blocking solution for 3 min for blockage of endogenous alkaline phosphatase. After washing with PBS, the slides were incubated with the primary anti-T lymphocyte (CD3) antibody for 30 min at room temperature, followed by incubation with the EnVision System (alkaline phosphatase-labeled polymer conjugated with goat anti-mouse and rabbit IgG polyclonal antibody) for 30 min at room temperature. The material was then washed and incubated with 1 mg/ml Fast Red in PBS containing tris-buffered naphthol for 15 min at room temperature. The slides were counterstained with Mayer’s hematoxylin and mounted in naphthol for 15 min at room temperature. The slides were washed with 1 mg/ml Fast Red in PBS containing tris-buffered naphthol for 15 min at room temperature. The slides were counterstained with Mayer’s hematoxylin and mounted in Faramount medium (Dako). Tonsils incubated or not with the primary antibodies were used as positive and negative controls, respectively. CD8+ T lymphocytes were stained brown while CD3+ T lymphocytes were stained red. Therefore, all cells showing a red color were considered to be CD4+ T lymphocytes.

**Histopathological analysis.** Slides stained with HE were analyzed by quantifying the cellular elements present in the dermis (histiocytes, lymphocytes, neutrophils and multinucleate giant cells) on a semiquantitative scale ranging from 0 to +, where 0 = absent, 1 = minimal, 2 = discrete, 3 = moderate, and 4 = intense [17,26]. Slides stained with Gomori’s methenamine silver were assessed regarding the presence of fungi in the lesions on a scale ranging from 0 to 4+, where 0 = absent, 1 = minimal, 2 = discrete, 3 = moderate, and 4 = intense [17,26]. Slides stained with HE-stained sections revealed a characteristic morphological pattern, irrespective of the clinical form of the disease shown by the patient, with the presence of a compound inflammatory infiltrate mainly consisting of histiocytes and multinucleate giant cells, including both foreign body and Langhans giant cells (Figure 1). Many histiocytes contained the fungus, a fact that conferred a peculiar morphology including an elongated nucleus and highly extended cytoplasm containing the yeast-like cells of *L. loboi*. Lymphocytes were present in the inflammatory infiltrate in discrete to moderate number and were scattered among histiocytes or formed small aggregates around blood vessels. Sometime, lymphocytes were also found diffusely distributed below the epidermis. Plasma cells were present in small numbers, while neutrophils were rarely observed.

**Characterization of the cell population present in the lesions.** CD68+ histiocytes predominated in the analyzed tissue samples, reaching a score of 4+ (Figure 2). The second most frequent cell type were CD3+ T lymphocytes, which were found scattered within the granuloma close to histiocytes and multinucleate giant cells, sometimes located below the epidermis forming a subepidermal mantle, sometimes forming small foci or aggregates around blood vessels. Most of these lymphocytes showed the CD4+ helper phenotype (Figure 3), with a CD4:CD8 ratio of approximately 3:2. Natural killer (NK) cells were frequently observed in the lesions, corresponding to the third most common cell type identified. These cells were found below the epidermis, scattered within the inflammatory infiltrate and close to histiocytes. Other cell types identified such as plasma cells and B lymphocytes were less frequent in the lesions. Plasma cells (CD79+) were present in the infiltrate in discrete numbers, while only a minimal number of B lymphocytes (CD20+) was observed. These cells were sparsely distributed and sometimes formed small aggregates close to blood vessels.

A similar cell population pattern in terms of both cell type and cell distribution within the inflammatory infiltrate was observed on patients with the localized and multicentric form of the disease.

### Table 1. Antibodies used for the characterization of the cell population in skin lesions obtained from patients with Jorge Lobo’s disease.

<table>
<thead>
<tr>
<th>Antibody Description</th>
<th>Clone</th>
<th>Manufacturer/Cat. No.</th>
<th>Dilution</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human anti-T lymphocyte polyclonal antibody, CD3+, produced in rabbits</td>
<td>–</td>
<td>Dako/A0452</td>
<td>1:75</td>
<td>T lymphocytes and subpopulations</td>
</tr>
<tr>
<td>Human anti-cytotoxic T lymphocyte monoclonal antibody, CD8</td>
<td>CB/144B</td>
<td>Dako/M7103</td>
<td>1:25</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>Human anti-B lymphocyte monoclonal antibody, CD20</td>
<td>L26</td>
<td>Dako/M0755</td>
<td>1:100</td>
<td>B lymphocytes</td>
</tr>
<tr>
<td>Human anti-B lymphocyte monoclonal antibody, CD79</td>
<td>JCB117</td>
<td>Dako/M7050</td>
<td>1:50</td>
<td>B lymphocytes and plasma cells</td>
</tr>
<tr>
<td>Human anti-natural killer cell monoclonal antibody, CD67</td>
<td>NK1</td>
<td>Lab. Vision/MS-136P</td>
<td>1:100</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>Human anti-macrophage monoclonal antibody, CD68</td>
<td>KP1</td>
<td>Dako/M0814</td>
<td>1:1000</td>
<td>Macrophage and monocytes</td>
</tr>
<tr>
<td>Peroxidase-labeled polymer conjugated with goat anti-mouse IgG antibody</td>
<td>–</td>
<td>Dako EnVision/K4001</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Peroxidase-labeled polymer conjugated with goat anti-rabbit IgG antibody</td>
<td>–</td>
<td>Dako EnVision/K4003</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Results**

**Histopathological analysis.** Histopathological examination of HE-stained sections revealed a characteristic morphological pattern, irrespective of the clinical form of the disease shown by the patient, with the presence of a compound inflammatory infiltrate mainly consisting of histiocytes and multinucleate giant cells, including both foreign body and Langhans giant cells (Figure 1). Many histiocytes contained the fungus, a fact that conferred a peculiar morphology including an elongated nucleus and highly extended cytoplasm containing the yeast-like cells of *L. loboi*. Lymphocytes were present in the inflammatory infiltrate in discrete to moderate number and were scattered among histiocytes or formed small aggregates around blood vessels. Sometime, lymphocytes were also found diffusely distributed below the epidermis. Plasma cells were present in small numbers, while neutrophils were rarely observed.

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A similar cell population pattern in terms of both cell type and cell distribution within the inflammatory infiltrate was observed on patients with the localized and multicentric form of the disease.
In the present study, we have characterized the mononuclear cell population present in the cutaneous lesions of patients with Jorge Lobo’s disease to better understand the host-parasite relationship in this mycosis. Histopathologically, the mycosis was characterized by a generally poor organization and a predominance of histiocytes and multinucleate giant cells, in addition to large numbers of fungi, many of them appeared morphologically nonviable. Lymphocytes, present in discrete to moderate numbers, were scattered among histiocytes or formed small foci.

Studies on the mononuclear cell population present in Jorge Lobo’s granuloma are scarce and usually conducted on a small number of patients. In the present study we characterized the cell population on the granulomatous lesions of 15 patients. Histiocytes were the most frequent cells observed, which were identified based on their morphological characteristics using HE staining as well as by the use of the monoclonal KP1 (CD68) antibody. The KP1 antibody recognizes an intracellular glycoprotein present in the lysosomes of cells of the monocyte/macrophage line, which persists throughout maturation of these cells and whose concentration is increased in activated and mature macrophages [15,19,28]. This antibody also labels multinucleate giant cells since they contain large numbers of lysosomes.

This study suggests that the fungi were phagocytized by histiocytes, which often formed aggregates, leading to the formation of foreign body giant cells. Many of these cells underwent a process of maturation, giving origin to Langhans multinucleate giant cells. We found a predominance of foreign body-type giant cells over Langhans cells, a characteristic finding mostly in granulomas caused by foreign body [2].

Similar to other studies [3,9,17], we only found a minimal number of neutrophils in most lesions examined. The experimental studies on BALB/c mice have shown that neutrophils are present in large numbers during the initial (24 h) and final (18 months) phases of the infectious process, while they are rare during the intermediate phases [12]. It is possible that the fungus L. loboi stimulates neutrophil migration, but this stimulus is not constant and, therefore, the effective participation of neutrophils in this mycosis seems irrelevant.

Analysis of the lymphocyte population by immunohistochemistry revealed a moderate number of T lymphocytes, usually observed nearby histiocytes or multinucleate giant cells or even around blood vessels. Most of these cells consisted of the T-helper subpopulation (CD4+). Although T lymphocytes were found to be distributed in a diffuse manner, the proximity between these lymphocytes and histiocytes may favor interaction between these cells, thus stimulating antigen recognition and the participation of cytokines in this event, which could lead to the activation of these macrophages.

In the present study, CD8+ T lymphocytes were also observed in the lesions at a CD4+/CD8+ T cell ratio of 3:2. These cells were located closely to helper T lymphocytes and histiocytes, but no cellular arrangement, such as those observed in the tuberculoid leprosy granuloma, was observed. In leprosy, CD4+ T lymphocytes are found in the center of the granuloma, while CD8+ T lymphocytes are located at the periphery away from macrophages and the pathogenic agent [16]. In the case of Jorge Lobo’s disease, the distribution of T lymphocytes resembles that observed in lepromatous leprosy, where helper and cytotoxic lymphocytes are diffusely distributed among macrophages [16].
Since in lepromatous leprosy this lymphocyte distribution is associated with events related to the failure of regulatory mechanisms required to contain the pathogen, the same may also occur in Jorge Lobo’s disease, a mechanism that could also explain the large number of fungal cells found in the lesions.

Analysis of the presence of yeast-like cells in the lesions by HE staining showed that numerous fungal cells possessed only empty capsules with scarce intracytoplasmic content suggesting nonviable cells. The mechanisms by which macrophages destroy *L. loboi* are still unknown. It is known that the fungus contains constitutive melanin in its cell wall [24], which might confer resistance to reactive oxygen intermediates produced by macrophages. Melanized cells seem to be less susceptible to death caused by reactive oxygen intermediates and reactive nitrogen intermediates than non-melanized cells. This pigment seems to be associated with fungal virulence, resistance to microbial attack, and greater survival under stress conditions [6,25]. The microbicidal mechanisms by which macrophages disrupt *L. loboi* in vivo would, therefore, be of great interest.

NK cells were always present in all lesions, but in a lower proportion than CD8+ T lymphocytes. The role of NK cells in the defense mechanisms against fungi has been studied by some investigators. In cryptococcosis, Levitz et al. [10] demonstrated that these cells were able to inhibit the in vitro growth of *C. neoformans*. Similarly, in paracoccidioidomycosis, Jimenez & Murphy [7] reported that murine NK cells limited the in vitro growth of *P. brasiliensis* and suggested that these cells may play a defensive role in the initial phase of the infection. In Jorge Lobo’s disease, it is possible that NK cells produce IFN-γ which activates macrophages, and that the fungus *L. loboi* is disrupted by this mechanism.

In this study we determined the presence of plasma cells and B lymphocytes in cutaneous lesions based on the CD79 and CD20 markers. CD79 is a glycoprotein that appears during the initial stage of B lymphocyte maturation and persists until the final plasma cell stage [14], while CD20 is a protein solely expressed on B lymphocytes [13]. The number of plasma cells in cutaneous lesions was higher than that of B lymphocytes, and the former were sometimes observed close to T lymphocytes and histiocytes or scattered within the infiltrate. The role of both cells in Jorge Lobo’s disease is unknown, but local secretion of specific antibodies is possible, which may act through mechanisms such as opsonization and activation of the complement system.

Studies on the characterization of the cell population present in cutaneous lesions of patients with Jorge Lobo’s disease are uncommon. Esterre et al. [3], analyzing lesions from three patients, observed a predominance of macrophages, few CD3+ T lymphocytes, most of them with a T-helper phenotype, and the absence of B lymphocytes. In a recent study, Ribeiro-Rodrigues et al. [20] phenotypically assessed the cell population of one patient with Jorge Lobo’s disease and found an absolute predominance of histiocytes (CD68+) and the absence of T (CD3+) and B (CD20+) lymphocytes.

This study showed that the distribution of mononuclear cells in cutaneous lesions follows the frequency: histiocytes (CD68+) > CD3+ T lymphocytes > CD4+ > CD8+ T lymphocytes > NK cells (CD57+) > plasma cells (CD79+) > B lymphocytes (CD20+). Based on the findings of a large number of fungi in the lesions and the disorganized cell arrangement in the granuloma, we hypothesize that patients with Jorge Lobo’s disease could have immunoregulatory disturbances, which are likely to be specific and quite possibly responsible for the lack of control over the pathogen. Further studies are necessary to analyze the in situ participation of key cytokines on cell containing fungal elements to better understand the pathogenesis of this mycosis.
References


