

Lacaziosis: immunohistochemical evaluation of elements of the humoral response in cutaneous lesions

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ABSTRACT

Lacaziosis is a cutaneous mycosis caused by the fungus *Lacazia loboi*, described in different countries of Latin America and prevalent in the Amazon region. The ineffective immune response against the agent seems to be related to a Th2 pattern of cytokines. There are few reports exploring elements of the humoral response in these lesions. Our aim was to investigate some elements focusing on B cells, plasma cells and local expression of IgG and IgM antibodies. Forty skin biopsies of lower limbs were selected. The diagnosis of lacaziosis was based on direct mycological examination and histological analysis. The visualization of fungal cells was improved by using Gridley's staining. An immunohistochemical protocol was performed to detect the expression of B cells, plasma cells, IgG and IgM. A double staining was performed to explore the presence of yeasts in the cytoplasm of keratinocytes, using an anti-AE1 AE3 antibody over Gridley's staining. The inflammatory infiltrate consisted of macrophages, multinucleated giant cells, lymphocytes, and fibrosis. Fungal cells were frequent in the stratum corneum and in both, the dermis and, in 50% of the specimens, also in the epidermis. Cells expressing IgG were more abundant when compared to cells expressing IgM. B cells and the presence of IgG might indicate that the humoral response promotes a Th2 immune response resulting in an anti-inflammatory phenotype. Our results lead us to suggest a possible role of B cells and immunoglobulins in the mechanisms of lacaziosis pathogenesis.

KEYWORDS: Lacaziosis. Humoral response. Skin. Human lesions. Immunohistochemistry.

INTRODUCTION

Lacaziosis is a cutaneous mycosis caused by the fungus *Lacazia loboi*, described in different countries of Latin America and prevalent in the Amazon region¹. Lesions are frequently located on the arms, legs and ears and appear after a trauma caused by a sharp instrument or arthropod bites. Clinically, they are characterized as keloid, warty, nodular, ulcerated or plaque lesions. The local immune response has been explored, but some aspects of the immunopathogenesis are still poorly understood^{2,3}.

Studies regarding the cellular immune response in lacaziosis lesions demonstrated the presence of macrophages and an intense expression of TGF- β , a fibrosis-associated cytokine, and the absence of well-organized granulomas⁴. The ineffective immune response against the agent seems to be related to a Th2-type cytokines pattern and the lesions comprise a clinical and immunological spectrum that varies from susceptible to resistant to treatment⁵. In addition, an expressive

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number of IL17-producing CD4+ T cells over TCD4+ Foxp3+ in lacaziosis skin lesions have been described, and it is suggested that the Th17 cytokine pattern may be present as an auxiliary mechanism for modulating the local immune response⁶. The cytotoxic function of CD8+ T cells was evidenced based on the detection of perforin and granzyme B and a role in the *in situ* immune response to eliminate the fungus was suggested. Similarly, by studying the cytotoxic cellular response in such lesions, we highlight and suggest an important role of these cytotoxic elements⁷.

Histologically, lacaziosis is already characterized by multifocal to coalescing dermal foci of granulomatous inflammation composed of macrophages, epithelioid cells, multinucleated giant cells, and often of large numbers of Gomori methanamine silver (GMS)-positive yeast cells, 6-12 µm in diameter, arranged individually or in chains and joined by tubular connections⁸. However, there are few reports exploring the presence of B cells, plasma cells and immunoglobulins in the lesions *in situ*. It is already known that patients with lacaziosis show increased serum IgG and IgM immunoglobulin levels⁹.

Our aim was to investigate some elements of the humoral immune response in such lesions focusing on the identification and quantification of CD20+ cells, plasma cells and local expression of IgG and IgM in the lesions.

MATERIALS AND METHODS

Forty skin biopsies of lower limbs from 40 lacaziosis patients were selected from the medical records of the Nucleo de Medicina Tropical, Universidade Federal do Para, Belem, PA, Brazil. The diagnosis was based on direct mycological examination and histological analysis. The visualization of fungal cells was improved by the Gridley's staining, based on the Schiff's reagent and aldehyde fuchsin¹⁰. An immunohistochemical protocol was performed as described: following deparaffinization in xylene and hydration in ethanol, antigen recovery was performed in a TRIS/EDTA solution pH 9.0 for 20 min at 95 °C. The primary antibodies anti-CD20 (Dako Cytomation, M755), anti-IgG (Dako Cytomation, A090), anti-IgM (Dako Cytomation, A091) and anti-plasma cells (CD138, Dako Cytomation, M7228) were applied at 1/400, 1/4000, 1/1000 and 1/500 dilutions, respectively. Following an overnight incubation at 4 °C, the secondary antibodies and a streptavidin-biotin peroxidase complex was applied (LSAB, Dako, Carpinteria, CA, EUA), for 30 min each. Then, the 3,3-diaminobenzidine tetrahydrochloride was used as the chromogen and the slides were counterstained with hematoxylin. We also performed a double immunohistochemistry to explore the presence of yeasts in the cytoplasm of keratinocytes, using

an anti-AE1 AE3 antibody (Dako Cytomation, M3515) followed by the Gridley's staining.

All reactions were performed with positive and negative controls. The negative ones were composed of isotype controls and the omission of the primary antibody. Immunolabeled cells were quantified in nine randomized fields. Statistical analysis was performed using the GraphPad Prism version 5.0 program (GraphPad Software, Inc., San Diego, California, USA), using the Mann-Whitney test.

Biopsies were selected according to the representativeness of the sample; whose size was enough to perform all the immunohistochemical procedures.

This research was approved by the Ethics and Research Committee of Faculdade de Medicina, Universidade de Sao Paulo, process N° 191/12.

RESULTS

The histological analyses of specimens showed epidermis with hyperplastic or ulcerated appearance. Fungal cells were frequently observed in the stratum corneum. The dermal changes were characterized by a clear band (grenz zone), of variable thickness separating the epidermis from the dermal infiltrate.

The inflammatory infiltrate consisted of macrophages, multinucleated giant cells, lymphocytes and fibrosis, visualized by hematoxylin-eosin staining. Fungal cells, visualized through the Gridley's staining, were observed in both, dermis and, in 50% of the specimens, also in epidermis, when the double staining was applied (Figure 1).

It was possible to observe immunolabeled cells with the anti-CD20, CD138, IgG and IgM in the inflammatory infiltrate of the lesions (Figure 2). The results on the quantification of positive cells were expressed as (median±SD) 12.44±82.24 for CD20; 21.33±139.10 for IgG; 3.55±18.97 for IgM and 5.33±58.75 for plasma cells (Figure 3).

DISCUSSION

Previous studies that characterized the inflammatory infiltrate in lacaziosis lesions evidenced the presence of both, plasma cells and B cells that allied to disorganized cells arrangements in the granuloma and the presence of many fungal cells, suggested that patients with lacaziosis present with immunoregulatory disturbances which are possibly responsible for the lack of containment of the pathogen¹¹.

Our results seem to evidence that there is participation of the humoral immune response in cutaneous lacaziosis

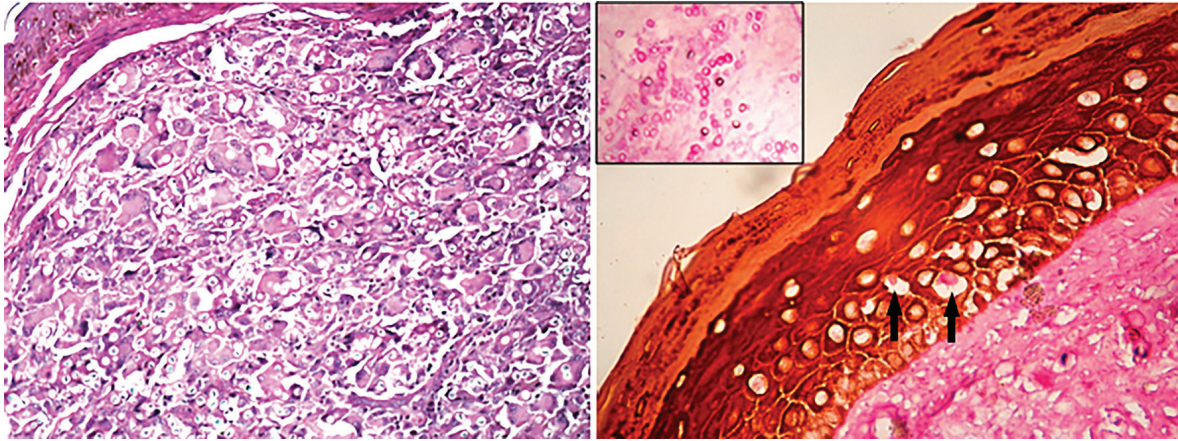


Figure 1 - Lacaziosis, skin lesions. A) Inflammatory infiltrate composed of macrophages, lymphocytes, epithelioid cells and multinucleated cells and a high number of fungal cells (Hematoxylin-Eosin, x 200); B) Double immunostaining evidencing the collocation of keratinocytes (in brown) and fungal cells (in purple) (x 400). Insert: single Gridley's staining evidencing fungal cells in the dermis (in purple).

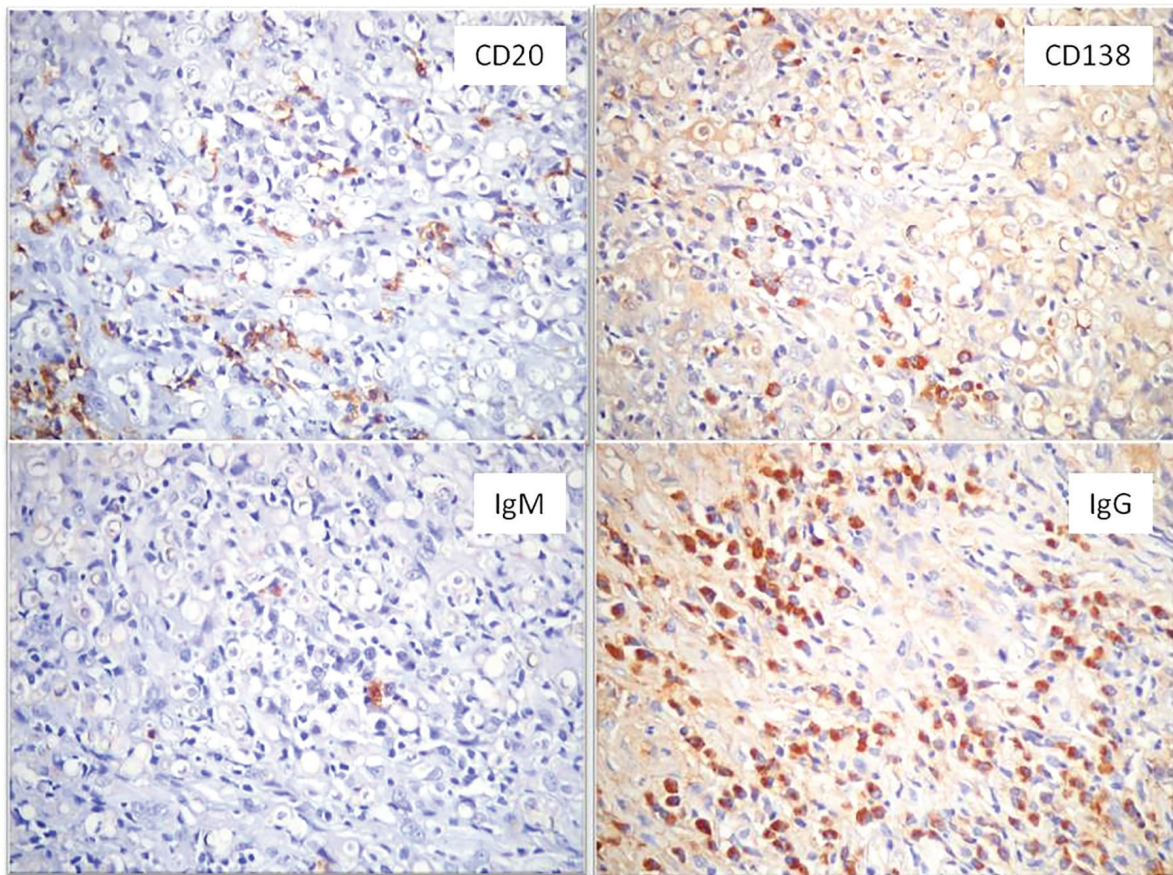


Figure 2 - Lacaziosis, skin lesions. Immunohistochemistry reaction visualized in brown. Upper left and right: High number of CD20+-B lymphocytes and CD138+-plasma cells in the inflammatory infiltrate, mixed with fungal cells in the granuloma; Lower left: Rare cells expressing IgM; Lower right: Intense expression of IgG in the inflammatory infiltrate (Streptavidin-Biotin HRP, x 200).

lesions. In fact, our immunohistochemistry results revealed the presence of many CD20-positive cells (B cells) and a high expression of IgG. In addition, this intense presence of both, CD20 and IgG positive cells was accompanied by other cells observed under routine microscopic analysis

characterized as plasma cells, macrophages, epithelioid cells, and giant cells as well. Plasma cells are characterized as large cells with abundant cytoplasm and an eccentric nucleus with heterochromatin in a characteristic cartwheel. A predominance of IgG expression over IgM ($p < 0.0001$)

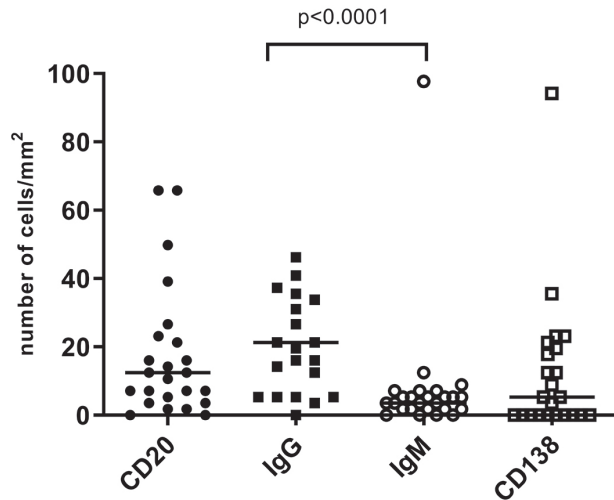


Figure 3 - Comparative analysis of immune-labeled cells expressing CD20, IgG, IgM and CD138. The comparison between cells expressing IgG and IgM evidenced a statistically significant difference ($p < 0.0001$). Values were expressed as the number of cells/mm². The median values \pm standard deviation were 12.44 \pm 82.24 for CD20; 21.33 \pm 139.10 for IgG; 3.55 \pm 18.97 for IgM and 5.33 \pm 58.75 for plasma cells.

was also observed. Interestingly, previous studies showed normal levels of IgG, IgM, IgA and serum proteins in patients with lacaziosis^{12,13}.

B cells with an immunoglobulin repertoire characterized mainly by IgG are results that might indicate that humoral response promotes a Th2 immune response and an anti-inflammatory phenotype in infected macrophages. This idea is based on the role of B cells in infectious diseases, especially those caused by intracellular parasites. For example, in experimental or human leishmaniasis, B cells appear to contribute to the persistence of parasites¹⁴⁻¹⁶. Miles *et al.*¹⁵, in a murine model, demonstrated that parasite-specific immunoglobulins failed to protect mice and promoted the persistence of parasites in B cell-deficient mice. Christensen *et al.*¹⁷, in the American tegumentary leishmaniasis model, reported patients with increased parasite transcripts in tissues, which were related to higher levels of immunoglobulins in serum and tissues.

Th1 response is required for clearing these intracellular parasites. The Th1 response, allied to Th17 cytokines production were described in lacaziosis as providing antifungal protection and the absence of a local Th1 response, with low levels of IFN- γ , may result in fungal persistence¹⁸.

Previous observations suggested that antibodies bound to fungi may function as opsonins and facilitate the macrophage-mediated phagocytosis of the microorganism, in addition to complement activation. Moreover, fungi with morphological features of unviability were observed in the inflammatory infiltrate. It was previously described

that lacaziosis is a disease of long evolution and lesions frequently present many fungal forms that are no longer viable^{19,20}. We did not perform viability tests in this study, however, as these authors^{19,20}, we considered the unviability of the fungi in the studied lacaziosis lesions based on their morphological features.

Our results lead us to suggest a possible role of B cells and immunoglobulin (IgG) in the mechanisms of lacaziosis pathogenesis. The observation in the present study of the predominance of cells expressing IgG might indicate that lacaziosis, as a chronic infectious disease, suffer a constant antigen stimulus, as previously considered. In frozen histological sections analyzed by immunofluorescence staining, deposits of C3, IgG and IgM were observed in the fungal wall of 100%, 91% and 18% of the lesions, respectively²⁰.

Finally, we were also able to detect the presence of fungal cells in the keratinocytes by double immunohistochemistry using an anti-AE1 AE3 antibody and Gridley's staining, corroborating previous observations²¹.

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AUTHORS' CONTRIBUTIONS

The authors' contributions were: substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work: AFA, GYFSN, AMSF, TCB, LKG, CP. Drafting the work or revising it critically for important intellectual content: WLT, JASQ, ACB, MNS, CP. Final approval of the version to be published: all. All authors read and approved the final manuscript. All authors made a substantial, direct and intellectual contribution to the work, and approved it for publication.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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