Article/Artigo

Cytokine expression in the duodenal mucosa of patients with visceral leishmaniasis

Expressão de citocinas na mucosa duodenal de pacientes com leishmaniose visceral

Kleber Giovanni Luz[1], Felipe Francisco Tuon[2], Maria Irma Seixas Duarte[3], Guilherme Mariz Maia[4], Paulo Matos[5], Ana Maria de Oliveira Ramos[6] and Antônio Carlos Nicodemo[2]

ABSTRACT

Introduction: Visceral leishmaniasis (VL) is a neglected tropical disease with a complex immune response in different organs. This pattern of organ-specific immune response has never been evaluated in the gastrointestinal tract. The aim of this study was to determine the *in situ* immune response in duodenal biopsies on patients with VL. **Methods:** A case-control study was conducted on 13 patients with VL in comparison with nine controls. The immune response was evaluated using immunohistochemistry, for CD4, CD8, CD68, IL-4, IFN-γ, TNF-α and IL-10. Histological findings from the villi, crypts and inflammatory process were analyzed. **Results:** All the cases of VL presented *Leishmania* antigens. No antigen was detected in the control group. The villus size was greater in the VL patients ($p < 0.05$). CD68 (macrophages) and CD4 levels were higher in the VL patients ($p < 0.05$). No differences in the expression of CD8, TNF-α, IL-10 or IL-4 were demonstrated. The number of cells expressing IFN-γ was lower in the VL patients ($p < 0.05$). **Conclusions:** Low levels of cytokines were found in the gastrointestinal tract of patients with VL. This pattern was not found in other organs affected by the disease. Immunotolerance of this tissue against *Leishmania* could explain these findings, as occurs with intestinal bacteria.

Key-words: Visceral leishmaniasis. Leishmaniasis. *Leishmania.*

RESUMO

Introdução: Leishmaniose visceral (LV) é uma doença tropical negligenciada com uma resposta imune complexa em diferentes órgãos. Este padrão de resposta imune órgão-específica nunca foi avaliada no trato gastrointestinal. O objetivo deste estudo foi determinar a resposta imune *in situ* em biópsias duodenais de pacientes com LV. **Métodos:** Um estudo de caso controle com 13 pacientes com LV foi comparado com 9 controles. A resposta imune foi avaliada por imunohistoquímica para CD4, CD8, CD68, IL-4, IFN-γ, TNF-α e IL-10. Achados histológicos nos vilos, criptas e processo inflamatório foram analisados. **Resultados:** Todos os casos de LV apresentaram antígenos de *Leishmania.* Nenhum antígeno foi encontrado no grupo controle. O tamanho do vilo foi maior em pacientes com LV ($p < 0,05$). CD68 (macrófagos) e CD4 estavam aumentados em pacientes com LV ($p < 0,05$). Nenhuma diferença foi demonstrada na expressão de CD8, TNF-α, IL-10 e IL-4. O número de células expressando IFN-γ foi mais baixo que no grupo controle ($p < 0,05$). **Conclusões:** Baixos níveis de citocinas foram encontrados no trato gastrointestinal de pacientes com LV. Este padrão não foi encontrado em outros órgãos acometidos pela doença. Uma imunotolerância do tecido contra *Leishmania* poderia explicar estes achados, como ocorre com as bactérias entéricas.

Palavras-chaves: Leishmaniose visceral. Leishmaniose. *Leishmania.*

1. Department of Infectious Diseases, Federal University of Rio Grande do Norte, Natal, RN, Brazil. 2. Department of Infectious Diseases, Medical School, University of São Paulo, São Paulo, SP, Brazil. 3. Laboratory of Infectious Diseases Pathology, Pathology Department, School of Medicine, University of São Paulo, São Paulo, SP, Brazil. 4. Hospital Infantil Varela Santiago, Natal, RN, Brazil. 5. Medical Laboratory of Pathology Getúlio Sales, Natal, RN, Brazil. 6. Hospital of Pediatrics, Federal University of Rio Grande do Norte, Natal, RN, Brazil.

***Address To*:** Dr. Kleber Giovanni Luz. R. Desembargador Túlio Bezerra de Melo 3631/1000, Candelária, 59065-200 Natal, RN, Brasil.

Phone: 55 11 3069-6530; Fax: 55 11 3069-7508

e-mail: luz@ufrnet.br

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INTRODUCTION

Leishmaniasis is a neglected tropical disease occurring in more than 80 countries throughout the world with increasing numbers of cases in some regions. The disease usually presents in two clinical forms: visceral and cutaneous. The latter is recognized as localized hard-to-treat ulcers. The visceral form is a chronic disease leading to hepatosplenomegaly with pancytopenia[1]. The immune pattern of visceral leishmaniasis (VL) associated with cytopenia is responsible for recurrent bacterial infection, which is a common cause of death in these patients.

Opportunistic bacteria gain access through the respiratory, the urinary and, especially, the digestive tract. The mechanism through which bacterial translocation occurs is generally explained by cytopenia. Nevertheless, a recent study showed that local immune factors may be the causes of these complications, including specific T cell depletion with cell deactivation and further cytokine imbalance[2]. This previous study demonstrated the existence of immune dissociation in the lungs. However, no studies have evaluated local immune conditions in the digestive tract, which is considered to be the organ most associated with bacterial translocation, because of the area and the major bacterial load. Furthermore, one study has revealed that 12% of patients with VL show gastrointestinal symptoms[3], while other studies have demonstrated impairment of vitamin A absorption among VL patients[4] and colitis in dogs[5].

The aim of the present study was to determine the *in situ* immune response in the gastrointestinal tract of patients with VL, by means of cell/cytokine evaluation by means of histopathological findings.

METHODS

Cases

A case-control study was conducted among children with VL who were diagnosed through findings of amastigotes in the bone marrow, in association with typical clinical findings. In Brazil,

VL is caused by *Leishmania infantum chagasi*, and all cases included in this study were from the city of Natal, in northeastern Brazil, and were recruited between January 2003 and January 2004. All the patients underwent endoscopy with duodenal biopsy by means of Watson's capsule, before the treatment for VL. The control group included children who underwent endoscopy because of gastrointestinal symptoms but who showed normal examinations for VL.

Histology

The duodenal tissue was fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned to micron thicknesses and stained with hematoxylin and eosin. Semi-quantitative methods were used to evaluate the size of the villi (0 = normal to 3 = atrophic), crypt characteristics (0 = normal to 3 = hyperplasia with hypertrophy four times normal values) and the presence of an inflammatory process (0 = normal to 3 = significant inflammatory process). The villus/crypt ratio was also analyzed.

Immunohistochemical detection of cells, inflammation phenotype and cytokines

The *in situ* immune response was studied through cytokine analysis and immunohistochemical study of phenotypic markers. The paraffin-embedded duodenal biopsies were resected and 5μm sections were mounted on sylane-treated slides. The sections were subjected to immunohistochemical processing with monoclonal antibodies, using the streptavidin-biotin peroxidase method with an endogenous biotin blocking system (Dako, Carpinteria, CA, USA) with modifications as described elsewhere[6]. Positive and negative control biopsies were used to avoid bias. The following monoclonal antibodies for cell phenotypes and cytokines were used in this process: CD4 (M834/Dako, Denmark), CD8 (M7103/Dako, Denmark), CD68 (M786/Dako, Denmark), IL-4 (AB204/R&D Systems, Minneapolis, MN, USA), IL-10 (MAB 217/R&D Systems), TNF-α (IP300/Genzyme, UK) and IFN-γ (IP500/Genzyme, UK).

The presence of *Leishmania* in the tissue was determined by means of antigen detection using immunohistochemistry as previous described[2]. We classified the results as positive or negative. No quantitative method was used.

Data analysis

Quantitative estimates of the different cell subsets in each biopsy were analyzed according to the density of the labeled cells for each stain (immunohistochemistry), using a grid scale, with 10 × 10 subdivisions in an area of 10mm^2, to count fields under high magnification (×400) in at least 10 fields, as described elsewhere[7].

The cytokine and immunolabeled cell medians in the control and VL tissues were compared by means of the nonparametric Mann-Whitney test. We took into consideration all differences in which the likelihood of similarity ($p < 0.05$) was significant. The chi-square test or the Fisher exact test was used, as appropriate.

Ethical

This study was approved by the Ethics Committee of Hospital das Clinicas, Federal University of Rio Grande do Norte. Cases with immunodeficiency, comorbidities and previous treatment were excluded.

RESULTS

Thirteen patients with VL were evaluated and nine patients were included in the control group. The median age was 3.14 years (range: 1-9) in the VL group and 7.6 years (range: 3-12) in the control group ($p < 0.05$). *Leishmania* antigens were detected in all patients with VL and none in the control group. No complication occurred following the endoscopic biopsy.

The results relating to villus size, crypts, villus/crypt ratio and inflammation are described in **Table 1**. The villus size was greater in the VL patients ($p < 0.05$).

The number of cells expressing CD68 (macrophages) and CD4 was higher in the patients with VL ($p < 0.05$). No difference in CD8 expression was demonstrated. The number of cells expressing cytokines was also evaluated, but no differences in IFN-γ, TNF-α, IL-10 or IL-4 expression were found.

The number of cases expressing IFN-γ was significantly lower among the patients with VL ($p = 0.023$). The number of cells expressing IL-10 in the VL group was similar to the number in the control group, and this was also found in relation to TNF-α and IL-4.

TABLE 1 - Case control study of patients with visceral leishmaniasis, evaluating the in situ immune response.

	Cases		Controls		
Data/cases	Mean	SD	Mean	SD	P value
Villus size (grade)	1.08	0.28	0.56	0.53	0.009
Crypt (grade)	0.54	0.52	0.56	0.53	0.94
Villus:crypt (ratio)	1.15	0.38	0.67	0.50	0.015
Inflammation (grade)	1.92	0.28	0.78	0.83	0.077
CD68 (cell/mm^2)	81.15	34.58	36.67	28.61	0.006
CD4 (cell/mm^2)	230.38	174.40	137.11	218.10	0.038
CD8 (cell/mm^2)	53.23	40.76	39.33	37.91	0.57
IFN-γ (cell/mm^2)	1.85	6.65	2.00	2.82	0.826
IL-10 (cell/mm^2)	13.62	20.30	30.22	32.14	0.143
IL-4 (cell/mm^2)	0.00	0.00	0.00	0.00	1
TNF-α (cell/mm^2)	2.42	5.90	0.24	0.44	0.3

DISCUSSION

The presence of *Leishmania* in intestinal tissue has been recognized since the time of the first cases of this disease and Muigai et al described the pathological characteristics very well[4]. Here, we described some *in situ* aspects of the disease that had not been shown previously. The increased numbers of macrophages in the intestinal tissue was not a new discovery, nor was the lymphoid infiltration (CD4 and CD8 cells). The morphological findings regarding villi, crypts and inflammation were also compatible with previous studies. The villi may be atrophic or hypertrophic, but are found to be normal in most cases, including in animal studies[8]. These data explain why chronic diarrhea and malabsorption are uncommon in immunocompetent patients with visceral leishmaniasis[9].

The findings of increased numbers of macrophages and CD4 cells without differences in cytokine expression between patients with VL and controls showed a different aspect of the immune response. This pattern was different from descriptions in other organs, such as the liver, spleen, kidney and lung[10]. The increased expression of IL-4 and

IL-10 with lower IFN-gamma is associated with an old concept of Th2 pattern of immune response. Nevertheless, localized cutaneous leishmaniasis caused by *Leishmania (Viannia) braziliensis* is the best concept of Th1 pattern. Despite these theories, the lack of difference in IL-4, IL-10 and TNF-α expression associated with lower levels of IFN-γ could be explained as an immunotolerant pattern, previously called a state of anergy[11].

The gut is considered to be an organ with an immune privilege, because of the confusing interplay between microorganisms, antigens and the intestinal epithelial barrier. The intestinal barrier can secrete immunoregulatory mediators that promote the generation of tolerogenic antigen-presenting cells, phagocytic innate immune cells and regulatory cells of the adaptive immune system. This complex interaction maintain the gut homeostasis[12].

The presence of *Leishmania* did not change this microsystem and the tolerance of the immune response enabled survival of this parasite for long periods without any inflammatory process. A detailed evaluation of the innate immune response is necessary to confirm this theory. NK cell, CD4CD25fox3+ cell and apoptosis evaluation are simple analyses that could bring out new concepts regarding immunotolerance in visceral leishmaniasis, with differences in relation to other organs.

Our study showed some limitations. The number of patients was too small to determine differences in some variables in relation to cytokine expression. The immune study was restricted to the duodenal mucosa. Studies in different parts of the gastrointestinal tract, such as the large intestine, should be performed because of their specific histological characteristics.

Dysregulation of the immunoregulatory network would lead to colitis, which has been described in dogs[5]. This would increase the percentage of intestinal signs and symptoms, thereby increasing the risk of translocation and further septicemia in such patients.

Cytokine responses in the intestine (in this case, the duodenum) were largely absent and only small changes in villus/crypt structures could be observed, thus indicating that apart from infiltration of macrophages, the structure was largely normal. The only conclusion that can be drawn from the results presented is that duodenal biopsies from VL patients are not very different from those of controls, with the exception of cell infiltration and, possibly, increased villus size.

CONFLICT OF INTEREST

The authors are not part of any associations or commercial relationships that might represent conflicts of interest in the writing of this study (e.g., pharmaceutical stock ownership, consultancy, advisory board membership, relevant patents, or research funding).

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