Original Article

Increased serum levels of interleukin-6 in erythema nodosum leprosum suggest its use as a biomarker

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Abstract

Background: Erythema nodosum leprosum (ENL) is a frequent complication of multibacillary leprosy that can result in significant morbidity, including peripheral nerve damage and physical disability. The identification of possible serum markers could be a valuable tool for the early detection of ENL.

Aims: The purpose of this study was to evaluate selected serum mediators involved in the innate and adaptive immune responses to identify possible immunomarkers for ENL.

Methods: The levels of interleukin-2, interleukin-4, interleukin-6, interleukin-10, interleukin-17, interferon-γ, tumor necrosis factor, nitric oxide and anti-phenolic glycolipid-I antibodies were measured in the sera of leprosy patients with ENL [at the beginning of reaction (M0) and 1 month later (M1)], and then compared with the levels of the same markers in patients with untreated multibacillary leprosy without ENL (controls with leprosy: CTRL) and healthy individuals (healthy controls: CTRH).

Results: Significantly higher levels of serum interleukin-6 were observed in M0 than in CTRL. In addition, pairwise comparisons showed higher levels of interleukin-6 in M0 compared to M1. Levels of tumor necrosis factor were higher in M0 than in CTRL, with no significant difference between M0 and M1. There were no differences in the levels of interleukin-2, interleukin-4, interleukin-10, interleukin-17 or interferon-γ between groups. The CTRL group had higher levels of nitric oxide compared to M0 and M1. High levels of anti-phenolic glycolipid-I were observed in M0, M1 and CTRL than in CTRH.

Limitations: Three patients were not assessed at M1, decreasing the number of evaluated patients from 14 to 11.

Conclusion: High-serum levels of interleukin-6 were observed during ENL, primarily in patients with more severe reactions; levels decreased after specific therapy, suggesting a role for this cytokine in pathogenesis and its utility as an ENL biomarker. Further studies should explore whether interleukin-6 could also be used as a predictive marker for ENL or as a specific target for its treatment.

Key words: Biomarker, cytokines, erythema nodosum, interleukin 6, leprosy

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Plain Language Summary

Leprosy is an infectious disease that affects nerves and skin. Some leprosy patients can suffer a kind of systemic inflammation known as erythema nodosum leprosum (ENL), that is characterized by the presence of painful nodules in the skin, fever, malaise and pain in the muscles and bones and may cause damage in nerves. Thus, the authors conducted a study in order to find some protein in the blood that could be used to help in early diagnosis of the condition. The study was carried out at Lauro de Souza Lima Institute, in Bauru, Brazil. It included leprosy patients with and without ENL and healthy individuals. The presence of some proteins that control the immune response, called cytokines, was assessed in the blood and the authors found that one protein, called interleukin-6 (IL-6), was increased only in the blood of patients with ENL. The authors suggested that IL-6 could be used as a marker for ENL.

Introduction

Erythema nodosum leprosum (ENL), or type 2 reaction, is an acute inflammatory complication that overlaps the chronic and insidious evolution of multibacillary leprosy and represents an important cause of morbidity and physical disability. ENL occurs mainly during or after treatment with multidrug therapy, but there are also cases in which it is the first manifestation, even before a diagnosis of leprosy.[1]

ENL affects patients with lepromatous leprosy and borderline lepromatous leprosy, known as multibacillary patients. It is characterized by the sudden appearance of papules, nodules, and painful plaques in the skin, which can progress to pustules and blisters with subsequent ulceration and necrosis in the most severe cases. Systemic manifestations can occur, including fever, malaise, generalized lymphadenopathy, pain in the muscles and bones, arthritis, iritis, iridocyclitis, orchitis, epididymitis, glomerulonephritis and neuropathy. Edema of hands, feet and face has also been observed.[1]

Episodes of ENL may either regress rapidly or persist for years, depending on the inflammatory response of individual patients. Thus, early diagnosis and appropriate management are essential to minimize clinical manifestations and alleviate patient suffering thereby providing a better quality of life.

The histopathology of acute lesions of ENL shows neutrophilic infiltration overlapping the chronic lepromatous pattern often associated with vasculitis, as well as the presence of fragmented bacilli.[2] The pathogenesis of ENL seems to be related to a predominantly Th2 cytokine profile and the presence of immune complexes.[3] Increased expression of mRNAs encoding interleukin-6, interleukin-8 and interleukin-10 and sustained expression of mRNAs encoding interleukin-4 and interleukin-5 have also been reported.[2,4] These cytokines are related to neutrophil chemotaxis, antibody production and reduced cell-mediated immune responses.

In contrast, other studies have demonstrated a role for Th1 responses in ENL through the expression of mRNAs encoding interferon-γ, tumor necrosis factor and interleukin-12.[3,5,6] More recent studies have also indicated the involvement of Th17 cells with elevated levels of interleukin-17 in culture supernatants and skin lesions;[7-9] however, no significant change in serum levels of interleukin-17 was observed in ENL.[10-12]

Given the importance of finding clinical and laboratory markers that recognize both ENL patients and those at increased risk of ENL development, the objective of our study was to evaluate serum mediators of innate and adaptive immune responses, including both pro and anti-inflammatory cytokines, to identify laboratory markers for ENL.

Methods

Subjects

Fourteen leprosy patients classified according to Ridley and Jopling (seven lepromatous leprosy and seven borderline lepromatous leprosy, twelve males and two females, mean age 56 years), with confirmed diagnoses of ENL by clinical and histopathological evaluations, were enrolled before initiating specific treatment for ENL.[13] Among these patients, eleven were evaluated again 1 month later (after recovery). In addition, sixteen patients with multibacillary leprosy but not treated with multidrug therapy and without ENL reactions (controls with leprosy: CTRL, seven lepromatous leprosy and nine borderline lepromatous leprosy, twelve males and four females, mean age 50 years) and fifteen healthy individuals (healthy controls: CTRH, eleven males and four females, mean age 51 years) were also recruited. The clinical status of ENL patients was evaluated according to Walker *et al.*[14] Briefly, mild reaction was defined as 1–10 ENL skin lesions with a little pain on palpation, no fever and absence of systemic signs and symptoms; moderate as 11–20 ENL skin lesions that were painful on palpation, fever between 37.6 and 38.5°C, edema of hands, face or feet at two sites, with pain or tenderness in lymph-node chains; and severe as 21 or more painful ENL skin lesions, ulcerated lesions involving a large area of the tegument, edema of hands, face and feet, high fever (>38.5°C), generalized involvement of lymph node chains and systemic signs and symptoms. Clinical characteristics of the ENL patients are shown in Table 1. Patients and controls were recruited at Lauro de Souza Lima Institute, Bauru, and in the Family Health Program, Rondonópolis, Brazil.

Table 1: Detailed description of erythema nodosum leprosum patients (M0) participated in the study

Patient/clinical form	Sex	Age (years)	ENL *ab initio*	ENL x MDT	ENL post-MDT	ENL severity	ENL post-M1*
1/BL	Male	57		X		Mild	X
2/LL	Male	40			X	Severe	X
3/BL	Male	64		X		Moderate	X
4/BL	Male	66		X		Severe	X
5/LL	Male	22			X	Severe	X
6/BL	Male	74			X	Moderate	X
7/BL	Male	56			X	Moderate	
8/BL	Male	56	X			Severe	•
9/BL	Female	51			X	Moderate	X
10/LL	Male	62		X		Mild	X
11/LL	Male	50			X	Severe	
12/LL	Female	76	X			Moderate	X
13/LL	Male	65	X			Moderate	X
14/LL	Male	50		X		Moderate	X

*After M1, the patients were followed up for 2 years. BL: Borderline lepromatous, LL: Lepromatous leprosy, ENL: Erythema nodosum leprosum, MDT: Multidrug therapy, M0: Moment of reaction of ENL, M1: 1 month postreaction, •: Death

Ethical statement

The study protocol was approved by the ethics committee of Lauro de Souza Lima Institute (number 1,824,819). Written informed consent was obtained from all participants after they were provided with a complete description of the study.

Blood collection

Peripheral blood (10 mL) was collected from each participant to obtain serum, which was aliquoted and stored at −80°C. For patients with ENL, blood was collected both at the beginning of the reactional episode, before of the specific treatment for the reaction (M0) and 1 month later (M1).

Quantification of cytokines

Serum levels of interleukin-2, interleukin-4, interleukin-6, interleukin-10, interleukin-17, interferon-γ and tumor necrosis factor were measured by flow cytometry using Th1/Th2/Th17 cytometric beads array kits according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Briefly, 50 µL of cytokine capture beads were incubated with 50 µL of serum and 50 µL of the detection antibody conjugated with fluorochrome phycoerythrin for 3 h at room temperature in the dark. The beads were washed by centrifugation and the samples were analyzed with a FACSCalibur flow cytometer (BD). Data were analyzed against a standard curve using FCAP Array software (BD). Serum levels of cytokines below the detection limit were expressed as 0 pg/mL.

Production of nitric oxide

Detection of serum nitric oxide was based on the measurement of nitrite (NO_2^-) by the colorimetric Griess reaction using n-(1-naphthyl) ethylenediamine and sulfanilamide. [15] After incubation for 10 min at room temperature, the reaction was read in an enzyme-linked immunosorbent assay microplate reader at 540 nm against a blank solution consisting of Griess reagent. The results (expressed as µmol) were generated by comparing the optical density of the reactions to a standard curve prepared for each assay.

Quantification of anti-phenolic glycolipid-I antibodies

Detection of IgM anti-phenolic glycolipid-I antibodies was performed using enzyme-linked immunosorbent assay as described by Brett *et al.* [16] Briefly, 96-well plates were coated with ND-O-BSA (natural disaccharide linked to bovine serum albumin, via an octyl) semi-synthetic antigen at 20 ng/mL (kindly provided by Dr. John Spencer, Colorado State University, USA) and blocked with 1% bovine serum albumin in phosphate-buffered saline (pH 7.4). Sera were diluted 1:500 in phosphate-buffered saline containing 0.1% Tween 20 and 10% normal goat serum (Gibco, Grand Island, NY, USA) and tested in duplicate. After incubation and washing, peroxidase-conjugated rabbit anti-human IgM polyclonal antibody (Dako Denmark A/S, Glostrup, Denmark) was added at a 1:10,000 dilution in phosphate-buffered saline containing 0.1% Tween 20 and 10% normal goat serum. After another incubation and washing, substrate [1% tetramethylbenzidine in dimethyl sulfoxide and 3.5 mM hydrogen peroxide in 0.1 M citrate buffer/acetate buffer, pH 6.0] was added and the reaction was stopped by the addition of 2.0 N H_2SO_4. The enzymatic activity was read in an enzyme-linked immunosorbent assay microplate reader using a 450-nm filter. Sera with optical density ≥ 0.2 were considered positive.[17]

Statistical analyses

Kruskal–Wallis one-way ANOVA followed by Dunn's multiple comparisons test was used to verify differences between groups. The Wilcoxon test was used for pairwise comparisons between M0 and M1. Data were analyzed using GraphPad Prism 5 software (GraphPad Inc., USA) and the differences were considered significant at $P < 0.05$.

Table 2: Quantification of interleukin-6 and tumor necrosis factor (pg/mL) in the serum of erythema nodosum leprosum patients at the moment of reaction (M0) and 1 month postreaction (M1) according to with clinical status of the patients

Patient/clinical form	ENL severity	IL-6/M0	IL-6/M1	TNF/M0	TNF/M1
1/BL	Mild	0.43	61.70	0	0
10/LL	Mild	116.21	90.64	50.37	66.97
Median	**Mild**	**58.32**	**76.17**	**25.19**	**33.49**
3/BL	Moderate	357.73	11.64	504.41	95.28
6/BL	Moderate	470.41	3.04	7.16	74.48
7/BL	Moderate	195.21	Not done	0.62	Not done
9/BL	Moderate	24.17	27.42	2.44	65.14
12/LL	Moderate	717.11	18.75	67.39	0
13/LL	Moderate	119.76	5.20	0	0
14/LL	Moderate	6.89	0	0	2.56
Median	**Moderate**	**195.21**	**8.42**	**2.44**	**33.85**
2/LL	Severe	28.18	6.72	18.92	31.64
4/BL	Severe	367.99	Not done (recurrent ENL)	99.33	Not done (recurrent ENL)
5/LL	Severe	638.50	Not done (recurrent ENL)	1.40	Not done (recurrent ENL)
8/BL	Severe	Not done	Death	0	Death
11/BL	Severe	400.57	0	16.73	0
Median	**Severe**	**384.28**	**3.36**	**16.73**	**15.82**

IL: Interleukin, TNF: Tumor necrosis factor, ENL: Erythema nodosum leprosum, BL: Borderline lepromatous, LL: Lepromatous leprosy

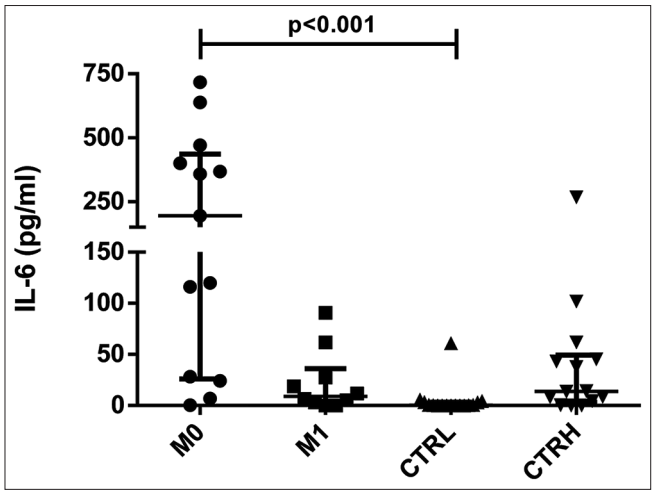

Figure 1a: Serum levels of interleukin-6 in erythema nodosum leprosum patients at the moment of reaction (M0) and 1 month postreaction (M1), leprosy patients without reaction (CTRL) and healthy individuals (CTRH). Bars represent median and interquartile range

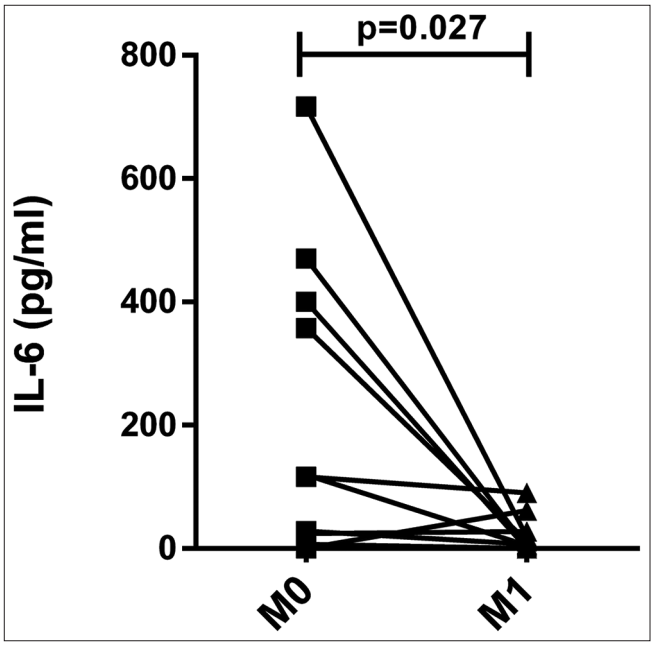

Figure 1b: Pairwise comparisons of serum levels of interleukin-6 in erythema nodosum leprosum patients at the moment of reaction (M0) and 1 month postreaction (M1)

Results

ENL patients had higher serum levels of interleukin-6 at M0 [median: 195.21 pg/mL, interquartile range: 28.18–440.57 pg/mL] compared to CTRL (0.37 pg/mL, interquartile range: 0.00–3.24 pg/mL) ($P < 0.001$) [Figure 1a]. Furthermore, the analyses of paired date ($n = 11$) showed that the production of interleukin-6 by ENL patients at M0 (117.99 pg/mL, interquartile range: 25.17–389.86 pg/mL) was higher than at M1 (9.18 pg/mL, interquartile range: 3.58–25.25 pg/mL) ($P = 0.027$) [Figure 1b]. Moreover, the level of interleukin-6 was higher in patients with severe ENL (median: 384.28 pg/mL) relative to those presenting with moderate (median: 195.21 pg/mL) and mild ENL (median: 58.32 pg/mL) [Table 2].

The serum levels of tumor necrosis factor were higher in both ENL patients at M0 (4.80 pg/mL, interquartile range: 0.16–42.51 pg/mL) and CTRH (78.15 pg/mL, interquartile range: 17.18–144.25 pg/mL) than in CTRL (0.00, interquartile range: 0.00–0.00 pg/mL) ($P = 0.041$ and $P < 0.001$, respectively) [Figure 2a]. When the results were analyzed with paired data for ENL patients, there was no significant difference between M0 and M1 (11.95 pg/mL, interquartile range: 0.61–42.51 pg/mL and 17.10 pg/mL, interquartile range:

0.00–66.51 pg/mL, respectively) [Figure 2b]. Tumor necrosis factor levels in patients with mild ENL (median: 25.19 pg/mL) were higher than in patients with severe (median: 16.73 pg/mL) and moderate ENL (median: 2.44 pg/mL) [Table 2].

There were no significant differences in the serum levels of interleukin-2, interleukin-4, interleukin-10, interleukin-17 and interferon-γ between M0 and M1, between M0 and CTRL, M0 and CTRH, M1 and CTRL, M1 and CTRH, and between CTRL and CTRH [Table 3]. Moreover, there was also no difference when the results were analyzed with paired data. It should be noted that serum levels of interleukin-2 and interleukin-4 at M0 and M1 were below the detection limits [Table 3].

The CTRL group had higher serum levels of nitric oxide (64.42 µmol, interquartile range: 51.97–73.50 µmol) than did M0 (32.76 µmol, interquartile range: 22.68–42.51 µmol) and M1 (32.20 µmol, interquartile range: 13.54–38.32 µmol) (P = 0.010 and P = 0.002, respectively) [Figure 3a]. The pairwise comparisons showed no significant differences between M0 (33.50 µmol, interquartile range: 22.71–49.58 µmol) and M1 (32.20 µmol, interquartile range: 13.54–38.32 µmol) [Figure 3b].

The levels of anti-phenolic glycolipid-I antibodies were higher in ENL patients at M0 (optical density: 0.274, interquartile range: 0.154–0.696), M1 (optical density: 0.319, interquartile range: 0.110–0.774), and CTRL (optical density: 0.393, interquartile range: 0.227–0.922) compared to CTRH (optical density: 0.034, interquartile range: 0.020–0.060) ($P < 0.001$, $P = 0.001$ and $P < 0.001$, respectively) [Figure 4a]. The pairwise comparisons revealed no difference between M0 (optical density: 0.380, interquartile range: 0.124–0.745) and M1 (0.319, interquartile range: 0.110–0.774) for anti-phenolic glycolipid-I antibodies [Figure 4b].

Table 3: Quantification of interleukin-2, interleukin-4, interleukin-10, interleukin-17 and interferon-γ (pg/mL) in the serum of erythema nodosum leprosum patients at the moment of reaction (M0) and 1 month postreaction (M1), leprosy patients without reaction (CTRL) and healthy individuals (CTRH). Results are expressed as median and minimum and maximum values

Patients/ controls	IL-2	IL-4	IL-10	IL-17	IFN-γ
M0	0.00 0.00-0.00	0.00 0.00-0.00	0.00 0.00-3.36	0.00 0.00-62.32	0.00 0.00-4.85
M1	0.00 0.00-0.00	0.00 0.00-0.00	0.00 0.00-4.04	0.00 0.00-23.72	0.00 0.00-0.00
CTRL	0.00 0.00-0.25	0.00 0.00-0.00	0.00 0.00-0.00	0.00 0.00-32.16	0.00 0.00-1.79
CTRH	0.00 0.00-57.15	0.00 0.00-39.43	0.00 0.00-39.69	0.00 0.00-0.86	0.00 0.00-0.64

IL: Interleukin, IFN: Interferon

Discussion

We have evaluated serum levels of interleukin-2, interleukin-4, interleukin-6, interleukin-10, interleukin-17, interferon-γ and tumor necrosis factor using Th1/Th2/Th17 cytometric beads array kits by flow cytometry, which has been widely used[18-20] to measure cytokines in small volumes of serum without losing sensitivity, accuracy or reproducibility.[21] Our results showed low levels of interferon-γ, interleukin-10 and interleukin-17 without a significant difference between M0 and M1, or between these time points and the control groups. In addition, interleukin-2 and interleukin-4 levels were below detection limits at M0 and M1. Therefore, these serum cytokines do not appear to be relevant to ENL, in contrast to the elevated levels of interleukin-6 and tumor necrosis factor during ENL episodes observed in this study.

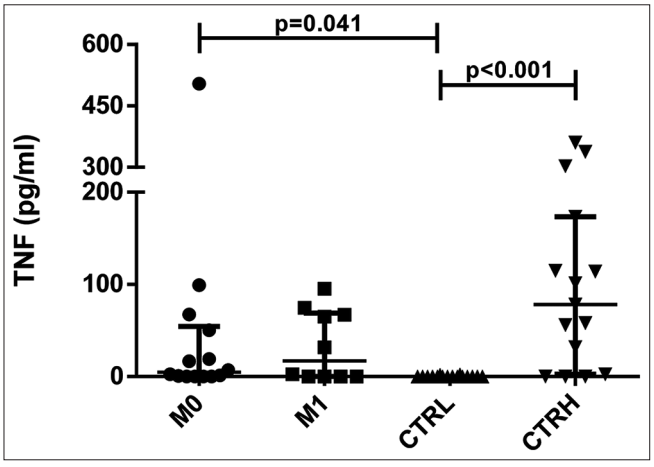

Figure 2a: Serum levels of tumor necrosis factor in erythema nodosum leprosum patients at the moment of reaction (M0) and 1 month postreaction (M1), leprosy patients without reaction (CTRL) and healthy individuals (CTRH). Bars represent median and interquartile range

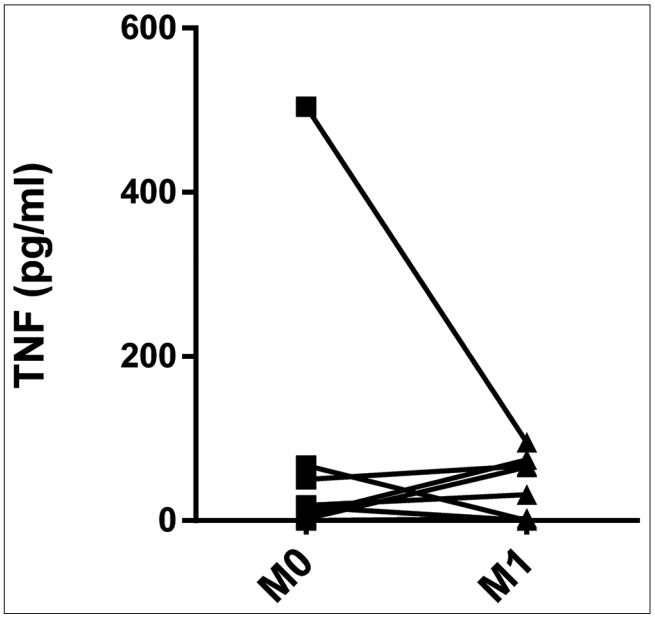

Figure 2b: Pairwise comparisons of serum levels of tumor necrosis factor in erythema nodosum leprosum patients at the moment of reaction (M0) and 1 month postreaction (M1)

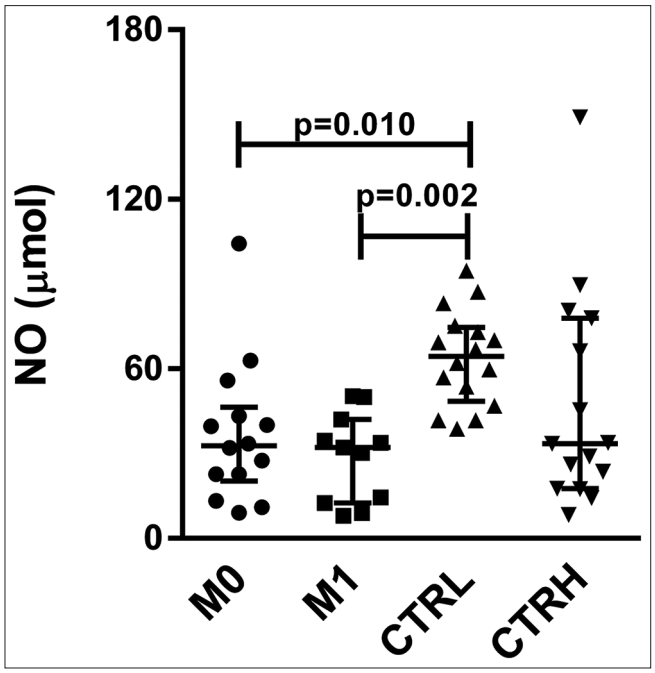

Figure 3a: Serum levels of nitric oxide in erythema nodosum leprosum patients at the moment of reaction (M0) and 1 month postreaction (M1), leprosy patients without reaction (CTRL) and healthy individuals (CTRH). Bars represent median and interquartile range

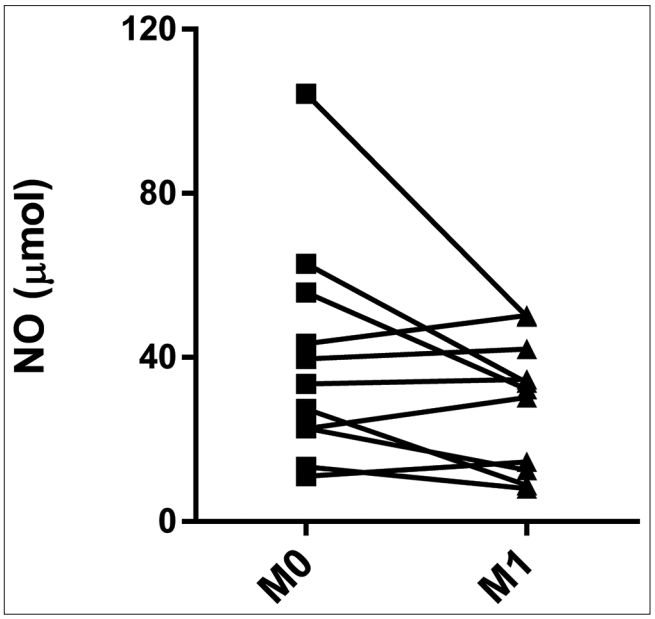

Figure 3b: Pairwise comparisons between M0 and M1

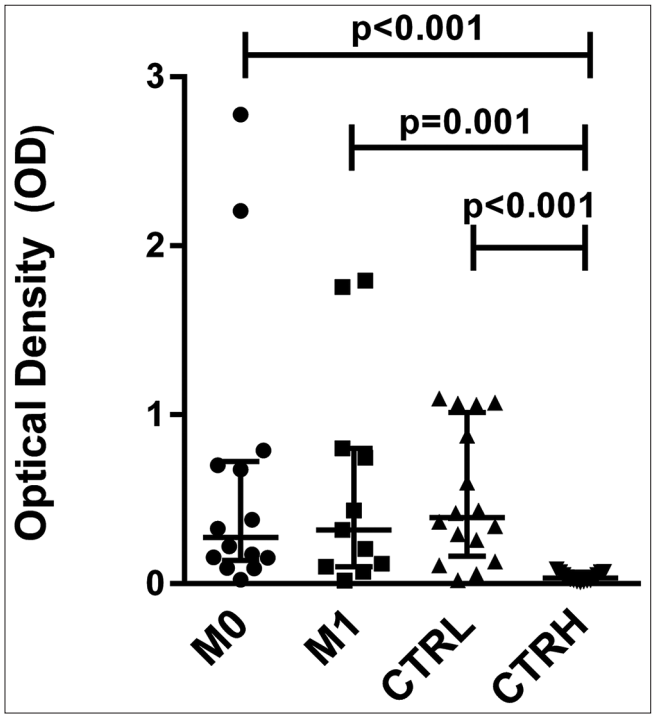

Figure 4a: Serum levels of antiphenolic glycolipid-I antibodies from erythema nodosum leprosum patients at the moment of reaction (M0) and 1 month postreaction (M1), leprosy patients without reaction (CTRL) and healthy individuals (CTRH). Bars represent median and interquartile range

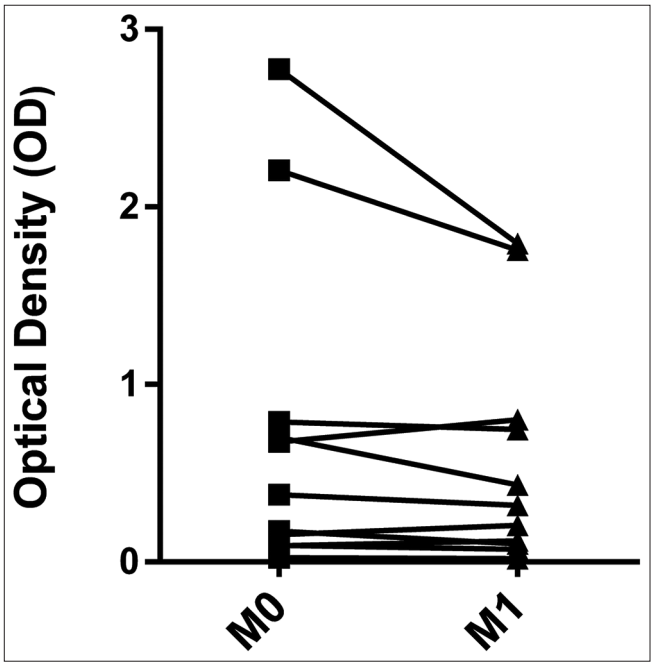

Figure 4b: Pairwise comparisons between M0 and M1

Of special interest, serum levels of interleukin-6 were higher in ENL at M0 than in M1 and the control groups (leprosy patients without reaction and healthy individuals). Results from pairwise comparisons revealed a significant difference in interleukin-6 levels between M0 and M1, suggesting that this cytokine has a role in ENL episodes and could be used as a marker for ENL in multibacillary leprosy patients. To date, there has been some research on laboratory parameters in ENL reactions; however, the results have been contradictory and with limited applicability.[17,22,23]

Studies evaluating serum levels of interleukin-6 in ENL have shown discordant results, with some reporting elevated levels,[24-26] similar to our findings, while others reported no association between interleukin-6 and ENL.[23,27] More recent studies have revealed the association of single-nucleotide polymorphisms in the *IL6* gene with ENL, with corresponding elevated serum interleukin-6 levels, consistent with our findings.[28,29] Considering the high levels of interleukin-6 during ENL reported in the literature[24-26,28,29] and its decrease after the remission of the reaction demonstrated by Villahermosa *et al.*[30] and our results, it is reasonable to propose an important role for interleukin-6 in ENL. To further support this hypothesis, we observed an association between interleukin-6 and the severity of ENL reaction, with higher serum levels of this cytokine in patients with severe ENL than in those with moderate or mild reaction .

Interleukin-6 is a proinflammatory cytokine that promotes the maturation and activation of neutrophils and stimulates the production of antibodies. Neutrophilic infiltration is involved in the initial phase of ENL reactions and decreases progressively over 9 days, representing the transition from the acute phase to the regressive phase of the reaction.[3,31] Furthermore, since interleukin-6 is synthesized in response to infections or tissue damage and stimulates the production of acute-phase proteins in hepatocytes, it is possible that the decreased serum levels of interleukin-6 observed at M1 are related to the regression of the inflammatory process.

Consistent with previous studies, our results revealed elevated serum levels of tumor necrosis factor in ENL patients at M0 in comparison to CTRL.[23,27,32-34] This finding, together with our previous observation of elevated tumor necrosis factor mRNA expression in ENL, suggests that this cytokine could also be used as an immunomarker for ENL.[35] However, there was no significant difference in serum tumor necrosis factor levels nor tumor necrosis factor mRNA expression between M0 and M1, although the values of this cytokine were slightly lower in M1.[35] Tumor necrosis factor levels have been shown to decrease in response to thalidomide, an effective drug in the treatment of ENL.[27,36,37] In this study, no patient was using thalidomide at M0, but six patients had used this drug between M0 and M1 and two of them had recurrent ENL, so they were excluded from the assessment at M1. Our results are in agreement with a study conducted by Haslett *et al.,* in which no difference was observed in tumor necrosis factor serum levels in ENL patients between the onset of the reaction and 28 days postreaction.[38]

In the present study, we also measured serum nitric oxide levels, since this metabolite could signal the participation of a cellular immune response (Th1), which has been suggested to be involved in ENL.[3,5,6] Our results showed higher levels of nitric oxide in CTRL than in M0, suggesting that nitric oxide does not actively participate in the reaction. We previously quantified nitric oxide in the supernatants of peripheral blood mononuclear cells culture from ENL patients and did not find a significant difference relative to healthy controls, consistent with findings from the present study. [35]

The serum levels of anti-phenolic glycolipid-I antibodies, an indicator of humoral immune response, were elevated in M0, M1 and CTRL, with no significant difference between reactional and non-reactional groups as observed by others.[17,39,40] However, conflicting observations have also been reported, showing lower levels in ENL, possibly due to the formation and deposition of immune complexes in tissues based on the pathophysiology of this reaction.[41,42]

Limitations

One limitation of this study was that two patients had recurrent ENL and one died; therefore, they were excluded from the assessment at M1 and the number of ENL patients was reduced from 14 to 11 at M1.

Conclusion

The follow-up of 13 ENL patients for two years showed that 11 patients had new episodes of ENL, reinforcing the importance of identifying biomarkers that may indicate early development of reaction.

Our results have shown that ENL patients have low serum levels of interferon-γ, interleukin-10 and interleukin-17 and high levels of interleukin-6 and tumor necrosis factor. More importantly, interleukin-6 was elevated only during ENL episodes (M0), reinforcing its role in the pathogenesis of this reaction and suggesting its utility as an immunomarker for ENL and a valuable indicator for the risk of ENL. Further studies should be conducted to address this possibility. An improved understanding of the actions of the cytokines and mediators during ENL can help develop new strategies for both treatment and prevention to avoid or reduce nerve damage, which severely impairs the quality of life of leprosy patients.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent.

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Conflicts of interest

There are no conflicts of interest.

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