Assessment of serum level of interleukin-1β and interleukin-12 in leprosy: impact of previous Bacillus Calmitte Guerin *vaccination*

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Assessment of serum level of interleukin-1β and interleukin-12 in leprosy: impact of previous *Bacillus Calmitte Guerin* vaccination

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Abstract Macrophages play an important role in attempt to eliminate mycobacteria, via production of cytokines, including interleukin-1, and interleukin-12. *Bacillus Calmitte Guerin* (BCG) vaccination, known to induce interleukin-1β in tuberculosis, was originally aimed at tuberculosis control, but it showed efficacy against leprosy. Our aim was to estimate serum levels of interleukin-1β and interleukin-12, in leprosy, and to assess the impact of previous BCG vaccination on their levels. Serum interleukin-1β and interleukin-12 p70 were estimated in 43 leprotic patients and 43 controls by enzyme-linked immunosorbent assay. Patients were grouped according to presence or absence of reactions, as well as bacillary load. Serum interleukin-1β was significantly higher in patients as compared to controls ($p = 0.047$), and was significantly different in patients' groups ($p = 0.036$); with significantly higher level in multibacillary patients, both non reactional and with erythema nodosum leprosum, compared with paucibacillary/non reactional patients ($p = 0.012$ and 0.049 respectively). A statistically significant higher interleukin-1β was found in BCG vaccinated paucibacillary patients as compared to unvaccinated patients ($p = 0.031$). Significantly elevated interleukin-12 was present in patients as compared to controls ($p < 0.001$), with no statistically significant difference comparing patients' groups. BCG vaccination showed stimulatory effect on monocytes only in the immunocompetent paucibacillary leprosy patients, as evidenced by higher Interleukin-1β in this group. Interleukin-1β was shown to have a pro-inflammatory role in multibacillary patients with or without erythema nodosum leprosum. Targeting interleukin-1β may be promising to control episodic refractory erythema nodosum leprosum. Interleukin-12 may be a general marker of active *Mycobacterium leprae* infection.

Keywords *Bacillus Calmitte Guerin* vaccine · Interleukin-1β · Interleukin-12 · Leprosy

Abbreviations

BB	Borderline borderline leprosy
BCG	*Bacillus Calmitte Guerin*
BL	Borderline lepromatous leprosy
BT	Borderline tuberculoid leprosy
CMI	Cell-mediated immunity
ELISA	Enzyme-linked immunosorbent assay
ENL	Erythema nodosum leprosum
HBV	Hepatitis B virus
HCV	Hepatitis C virus
IFN	Interferon
IL	Interleukin
LL	Lepromatous leprosy
MB	Multibacillary
M. avium	*Mycobacterium avium*
M. leprae	*Mycobacterium leprae*
p	Probability factor
PB	Paucibacillary
PNL	Pure neural leprosy
r	Correlation factor
RL	Reactional leprosy
SPSS	Statistical program for social science
SSS	Slit-skin smear examination
Th	T helper cells
TT	Tuberculoid leprosy

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Introduction

Leprosy is a chronic granulomatous disease caused by the bacteria *Mycobacterium leprae* (*M. leprae*) [30]; an obligatory intracellular pathogen that primarily affects the peripheral nerves and secondarily the skin, mucous membrane of the mouth and upper respiratory tract [3]. Host immunity to *M. leprae* determines the diversity of clinical manifestations seen in patients, from tuberculoid leprosy (TT) with robust production of T helper (Th)1-type cytokines, to lepromatous disease (LL), characterized by elevated levels of Th2-type cytokines [32]. Between those two polar forms lie the borderline forms of the disease, with the extent of the disease reflecting the balance between cell mediated immunity (CMI) and the bacillary load. These forms are liable to reactional leprosy (RL) type 1, which seems to be associated with a sudden increase in CMI against *M. leprae* antigens and is characterized by a predominantly type-1 cytokine profile [22, 27]. The RL type 2; erythema nodosum leprosum (ENL) type of reaction, which occurs in borderline lepromatous leprosy (BL) and LL patients, is a more systemic reaction than the previous and is immunopathologically more complex as well [26].

Macrophages play an important role in body's attempt to eliminate the lepra bacilli. When these cells encounter *M. leprae*, they produce cytokines, including interleukin (IL)-1, and IL-12. These cytokines then stimulate the number and activity of other macrophages [19].

Interleukin-1 is one of the first cytokines ever described. It is composed of two distinct proteins IL-1α and IL-1β [10]. Both IL-1α and IL-1β are produced by macrophages, monocytes and dendritic cells. They form an important part of the inflammatory response of the body against infection. IL-1β has many biological activities, including fever, anorexia, and leukocytosis. It also stimulates other cytokines, chemokines, and acute-phase reactants [24].

IL-12 is produced also by dendritic cells and macrophage and acts on T cells, thus forming a major link between innate and adaptive immunity [28]. It is a heterodimeric cytokine, encoded by two separate genes, IL-12A (p35) and IL-12B (p40), with no apparent homology. When they are co-expressed in the same cell, they form the biologically active IL-12 p70 heterodimer [15]. IL-12 is involved in the differentiation of naive T cells into Th1 cells. It is known as a T cell stimulating factor, which can stimulate the growth and function of T cells. IL-12 was shown to mediate enhancement of the cytotoxic activity of natural killer cells and cytotoxic T lymphocytes. Enhanced functional response was demonstrated by interferon (IFN)-γ production and killing of target cells [36].

Bacillus Calmitte Guerin (BCG) vaccination was originally aimed at tuberculosis control, but in fact, it appeared to be of effectiveness against leprosy; affording 40–50 % protection [16]. Van Crevel et al. [35] reported that live BCG induced production of proinflammatory cytokines, such as IL-1β, in tuberculosis patients and healthy individuals irrespective of their tuberculin skin status.

The aim of the study was to estimate serum levels of IL-1β and IL-12 p70, in leprosy, including RL and non RL, to gain further insight into their role in the immunopathogenesis of this chronic disabling disease. We also aimed to assess the impact of previous BCG vaccination on their levels.

Materials and methods

This study was conducted on 43 untreated leprotic patients attending at Dermatology and Leprosy El Qal'ah Hospital, Cairo, Egypt, and 43 healthy volunteers as a control group, after signing an informed consent, over a period of 10 months. The study was conducted according to the Declaration of Helsinki Principles, and was approved by the medical ethical committee of Ain Shams University. Patients were evaluated according to clinical examination, slit skin smear examination (SSS), and histopathological examination. Based on Ridley-Jopling classification [17], there were 11 patients with LL (25.6 %), 6 had TT (14 %), 9 had Borderline leprosy (20.9 %), 6 had RL type 1(14 %), 6 had E.N.L (14 %), and 5 patients had PNL (11.6 %). According to SSS, patients were classified into paucibacillary (PB) with negative SSS, and multibacillary (MB) with positive SSS [37]. BCG vaccination history during early infancy was taken, with checking of birth certificate for registration of compulsory vaccines according to the rules in Egypt. Examination of vaccination site was also done for the presence of the usual small, flat scar in vaccinated individuals.

The patients were categorized into 4 groups as follows. They were divided according to the presence or absence of RL at time of sample recruitment into Non RL (31 patients), and RL (12 patients). Non RL patients were further sub-divided into: PB/Non RL [including TT, borderline tuberculoid (BT) patients, and pure neural leprosy (PNL); 16 patients], and MB/Non RL [including borderline borderline (BB), BL, and LL cases; 15 patients]. RL cases were sub-divided into RL type1 group (6 patients) and ENL group (6 patients). In addition, 43 healthy age- and gender-matched subjects with negative hepatitis C virus (HCV) and hepatitis B virus (HBV) serology and negative tuberculin test (negative controls; to avoid possible impact of tuberculin positivity of controls on study outcome), from hospital staff members and personnel, were also included in the study, comprising the control group.

Blood samples were collected from both patients and controls, provided that all subjects were free of any other systemic disease. Patients who started antileprotic treatment or were on any kind of immunomodulatory or immunosuppressive therapy likely to alter the results of the study, such as systemic corticosteroids, were excluded. Blood was collected in sterile test tubes and centrifuged for 15 min at 50 g. Serum was separated and kept at−70 °C until used for estimation of IL-1β and IL-12 p70 by enzyme-linked immunosorbent assay (ELISA) by the RayBio® human ELISA kits, according to manufacturer guidelines. The assay limit is 6.3–400 pg/ml.

Statistical analysis of the collected data was done by IBM computer using SPSS (statistical program for social science) version 12 (SPSS Inc., Chicago, IL, USA). Quantitative data presented in medians and ranges were non-parametric. Kruskal–Wallis for multiple comparisons and Mann–Whitney test for comparisons between 2 groups were used for analytical statistics of such data. Bonferroni adjustment was used in case of multiple comparisons. Spearman correlation was used to measure the correlation between the quantitative variables. A probability "p" value of <0.05 was considered significant, while <0.001 was highly significant.

Results

The current study included 43 leprotic patients; 16 females (37.2 %) and 27 males (62.8 %). Their ages ranged from 15 to 65 years with a mean age of 35.74 years ± 11.23. Forty-three clinically free individuals served as controls; 16 females (37.2 %) and 27 males (62.8 %). Their age ranged from 25 to 54 years with a mean age of 37.7 years ± 10.2. No statistically significant difference was found between male and female patients, regarding either interleukins serum levels with median of 31 versus 28 pg/ml in IL-1β or median of 38 versus 48 pg/ml regarding IL-12 ($p = 0.449$ and 0.272 respectively). No correlation was detected between the measured serum levels of either IL-1 or 12 and the age of the patients ($r = -0.112$, $p = 0.474$ and $r = -0.172$, $p = 0.271$ respectively). SSS was negative in 16 patients (37.2 %), grouped as PB, while it was positive in 27 patients (62.8 %); grouped as MB. PB/Non RL category included 16 patients (37.2 %) [Including 6 patients (14 %) with TT, 5 (11.6) with BT, and 5 patients (11.6 %) suffering from PNL]. MB/Non RL category included 15 patients (34.8 %) [Including 3 patients (6.9 %) with BB, 1 (2.3 %) with BL, and 11 patients (25.6 %) with LL]. RL included 12 patients and were categorized into 6 patients (14 %) suffering from RL type1, and 6 patients with ENL (14 %).

Comparing leprotic patients with controls showed higher IL-1β level in patients ($p = 0.047$). Regarding the type of leprosy, IL-1β was found to be highest among LL type, while the lowest level detected was in PNL type. A statistically significant difference was found on comparing serum IL-1β level in the different categories of leprosy ($p = 0.036$) (Table 1). A statistically significant difference was found between both PB/Non RL and MB/Non RL patients (p value = 0.012), and also between PB/Non RL and ENL patients (p value = 0.049) using Mann–Whitney test.

Bacillus Calmitte Guerin vaccination was positive in 3/16 of SSS negative-(PB) patients (18.75 %); and in 9/27 of SSS positive-(MB) patients (33.33 %), with no statistically significant difference ($p = 0.865$). However, we found that IL-1β level was influenced by history of vaccination in PB patients (Table 2).

Regarding serum IL-12, it showed statistically highly significant elevated level in patients as compared to controls ($p < 0.001$). Serum IL-12 level in patients with negative SSS ranged from 20 to 68 pg/ml with a median of 40, while in MB cases, it ranged from 20 to 134 pg/ml with a median of 40, and no statistically significant difference was found ($p = 0.386$). A statistically significant difference was found on comparing serum IL-12 level in different types of leprosy (PB/Non RL, MB/Non RL, ENL and Type 1 reaction) with controls; ($p < 0.001$, $p < 0.001$, $p = 0.004$, and $p < 0.001$ respectively) using Mann–Whitney test (Table 3).

IL-12 level was not influenced by previous BCG vaccination in either PB patients or MB patients ($p = 0.176$ and 0.73 respectively) (Table 4). No correlation was detected between the serum levels of both IL-12 and IL-1 ($r = 0.147$, $p = 0.346$).

Discussion

Interleukin-1 is a potent proinflammatory cytokine that acts as an endogenous pyrogen. It mediates many inflammatory diseases by initiating and potentiating immune and inflammatory responses [11]. IL-1β is the prominent form of IL-1 and the amount of IL-1β mRNA found in activated cells is usually 10–50 fold greater than the α form. In addition, culture supernatant and body fluid contain more IL-1β than the α form [9].

Particular attention has focused on the role of IL-1 in T lymphocyte-dependent immune responses, which are essential for CMI to antigens, whether of microbial or neoplastic origin [2]. IL-1β was considered as a proinflammatory cytokine that mediates the inflammatory responses in leprosy, including leprosy reactions [12]. In accordance, significantly higher level was detected when comparing IL-1β level in patients and controls in the present study. Particularly, significantly higher level was

Table 1 Comparison between the different patients' subgroups using Kruskal–Wallis test, and comparison between the different patients' subgroups and controls using Mann–Whitney test, regarding IL-1β levels

Group (number)	IL-1β (pg/ml) In patients Median (range)	Significance (Kruskal–Wallis test)	IL-1β (pg/ml) in controls (43) Median (range)	Significance (Mann–Whitney test)
Paucibacillary/non reactional leprosy (16)	18 (2–88)			$p = 0.932$
Multibacillary/non reactional leprosy (15)	40 (16–88)	$\mathbf{p = 0.036}$*	**22(1–62)**	$p = 0.130$
Erythema nodosum leprosum (6)	39 (18–58)			$p = 0.130$
Type 1 reaction (6)	28 (18–40)			$p = 0.688$
All patients (43)	**30 (2–88)**		$\mathbf{p = 0.047}$**	

* $\mathbf{p = 0.036}$ indicates significant difference between patients' subgroups

** $\mathbf{p = 0.047}$ indicates significant difference between all patients' group versus control group

Table 2 Comparison between serum levels of IL-1β in BCG vaccinated versus non-vaccinated MB patients, as well as vaccinated versus non-vaccinated PB patients, using Mann–Whitney test

IL-1β (pg/ml)	BCG-positive		BCG-negative		U	*p* value
	Median	Range	Median	Range		
Slit skin smear-positive	31	22–40	39	8–88	11.5	0.798
Slit skin smear-negative	39	31–88	18	2–42	3.5	**0.031***

* $\mathbf{p = 0.031}$ indicates significant difference between BCG vaccinated versus non vaccinated paucibacillary patients (slit skin smear-negative)

Table 3 Comparison between the different patients' subgroups using Kruskal–Wallis test, and comparison between the different patients' subgroups and controls using Mann–Whitney test, regarding IL-12 levels

Group (number)	IL-12 (pg/ml)		Significance (Kruskal–Wallis test)	IL-12 (pg/ml) in controls (20)		Significance (Mann–Whitney test)
	Median	Range		Median	Range	
Paucibacillary/non reactional lepros (16)	40	20–94		**12.5**	**2–28**	$\mathbf{p < 0.001}$**
Multibacillary/non reactional lepros (15)	46	20–134	$p = 0.748$			$\mathbf{p < 0.001}$**
Erythema nodosum leprosum (6)	34	20–108				$\mathbf{p = 0.002}$***
Type 1 reaction (6)	39	30–66				$\mathbf{p < 0.001}$**
All patients (43)	**40**	**20–134**		$\mathbf{p < 0.001}$*		

* $\mathbf{p < 0.001}$ indicates highly significant difference between all patients' group versus controls

** $\mathbf{p < 0.001}$ and *** $\mathbf{p = 0.002}$ are highly significant and indicate significant difference respectively between patients' subgroups versus controls

Table 4 Comparison between serum levels of IL-12 in BCG vaccinated versus non-vaccinated MB patients, as well as vaccinated versus non-vaccinated PB patients, using Mann–Whitney test

IL-12 (pg/ml)	BCG-positive		BCG-negative		U	*p* value
	Median	Range	Median	Range		
Slit skin smear-positive	44	22–66	50	20–134	11	0.73
Slit skin smear-negative	28	28–40	40	20–94	9.5	0.176

shown in MB patients, whether non RL patients or with ENL, compared with PB/non RL patients. In agreement, Moubasher et al. [25] stated that LL patients showed significantly higher serum levels of IL-1β with significant positive correlations with bacterial index (BI). Moreover, Leal et al. [20] found elevated levels of IL-1β in LL/BL leprosy patients, associated with an exacerbated inflammatory response to the elevated BI. In addition, both type 1 and 2 RL patients showed significantly higher serum IL-1β in another report [8]. Immune complexes were reported to stimulate production of IL-1β, which may explain the elevated serum levels of IL-1β in ENL. Besides, IL-1β was found to be an inducer of Th2 responses and antibody production by B-cells; both occur in ENL [6].

IL-1β dysregulation can cause auto-inflammatory disease, often with cutaneous manifestations, including familial Mediterranean fever, Muckle-Wells syndrome, and TNF-receptor–associated periodic syndrome. Auto-inflammatory diseases tend to be episodic, associated with exogenous triggers, and respond to Anakinra; a recombinant IL1 receptor antagonist; IL-1ra [13]. In the context of leprosy, Fafutis-Morris et al. [14] found high IL-1ra in LL patients, which may indirectly, reflects high IL-1 [21]. Thus, it is recommended to correlate IL-1ra levels to IL-1 levels in the sera of leprosy patients with different clinical forms, as compared to controls. In addition, targeting IL-1β can be an area of future research to control episodic refractory ENL. Yet, Anakinra has been reported to reactivate pulmonary TB upon prolonged treatment [31]. Therefore, proper patients' selection and close monitoring is required for such treatment.

Mycobacterium tuberculosis and its cell wall components, as well as heat shock proteins from *M. tuberculosis*, *M. leprae*, and *M. bovis*, were found to induce mRNA for IL-1β in human monocytes [18]. Van Crevel et al. [11] reported that live BCG induced production of proinflammatory cytokines, including IL-1β, in tuberculosis patients and healthy individuals irrespective of their tuberculin skin status. In accordance, a statistically significant higher level of IL-1β was found on comparing levels in BCG vaccinated PB patients to those patients who were not vaccinated. However, previous BCG vaccination did not influence the disease category, or bacillary load, and did not show any influence on serum levels of IL-12, for further studies on larger populations. Sinsimer et al. [32] suggested that monocyte production of the pro-inflammatory cytokines, including IL-1, in response to *M. leprae* stimulation was absent or very low as compared to that in response to BCG stimulation.

Similar to our leprosy patients, comparing serum IL-12 level with controls, it was found to be higher in active pulmonary tuberculosis patients [1]; the prototype of mycobacterial infection in which BCG vaccination is used for prophylaxis. A variety of approaches were being pursued to increase the immunogenicity of BCG vaccine, including the addition of recombinant IL-12 protein as an adjuvant. Co-administration of plasmid-encoded IL-12 with DNA expressing the dominant *M. avium* 35,000 MW protein was found to enhance the immunogenicity of the DNA vaccine and was significantly more protective than BCG against a strain of *M. avium* which causes progressive infection [23]. The 35,000 MW protein was first identified as the homologue of the *M. leprae* major membrane protein I [34], which elicits either a strong CD4 T cell and/or antibody response in >90 % of leprosy patients across the leprosy spectrum [33]. These data implies the possible use of IL-12 as an adjuvant in vaccination against *M. Leprae* infection.

In the current study, a highly significant elevation of serum IL-12 was detected on comparing leprosy patients group to controls, as well as comparing different patients' subgroups to controls. In accordance with our results, De Paula et al. [7] observed a highly significant mean level of IL-12 in leprosy patients' serum. Regarding RL, type1 RL appears to be mediated via Th1 lymphocytes, and cells from reactional lesions express the proinflammatory cytokines IFN-γ and IL-12 [22]. In contrast, LL patients manifest disseminated infection, their T cells weakly respond to *M. leprae*, and their lesions express the type 2 cytokines, typical of humoral responses and immunosuppression of CMI. Here, the IL-12 levels should be extremely low [38]. However, the pathogenesis of ENL involves immune complex deposition, activation of complement and migration of neutrophils with release of IL-12 [5]. Thus, IL-12 seems to play a significant role in both types of leprosy reactions by orchestrating the triggering events that led to the development of these episodes [29].

Although IL-1 can act synergistically with IL-12 [4], our results regarding the positive correlation between the serum levels of IL-1 and IL-12 were not statistically significant. Further studies on larger number of patients can obviate a significant correlation between the mentioned cytokines.

In conclusion, IL-1β was shown to have a pro-inflammatory role in MB patients with or without ENL. IL-1β dysregulation can cause episodic auto-inflammatory disease, associated with exogenous triggers, probably including ENL. Targeting IL-1β can be an area of future research to control episodic refractory ENL. On the other hand, the overproduction of IL-12 in patients with leprosy infers that it is an important marker of active immune response to *M. leprae* antigen. Using IL-12 as an adjuvant in vaccination against *M. Leprae* infection could be promising in the context of leprosy prophylaxis. Previous BCG vaccination showed stimulatory effect on monocytes, in the immunocompetent PB leprosy patients, as evidenced by higher IL-1β in them. This infers that BCG vaccination for leprosy prophylaxis may be effective only in immunocompetent hosts, for further studies.

Limitations

The small number of patients in some of the patients' subgroups limits the statistical power, for further study on larger population of patients.

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