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Expression and Analysis of CXCL8 and CXCL10 Chemokines in Human Skin Lesions Infected with *M.leprae*

Krishan Dutta Rawat[1], Mamta Chahar[1], PVJ Reddy[1], Nalini Srivastava[2], U.D.Gupta[1], M. Natrajan[1], V.M. Katoch[3], Kiran Katoch[1] and D.S. Chauhan[1*]

[1]*National JALMA Institute for Leprosy & Other Mycobacterial Diseases Tajganj, Agra, U.P, India*

[2]*SOS in Biotechnology, Jiwaji University, Gwalior, M.P, India.*

[3]*Department of Health Research, Indian Council of Medical Research, Ansari Nagar, New Delhi, India*

*Corresponding author: Chauhan DS, Microbiology & Molecular biology Lab, National JALMA Institute for Leprosy and Other Mycobacterial Diseases, (ICMR) Tajganj, Agra-282001, Tel: (0562) 2331751-4; Fax: +91-562-2331755; E-mail: devchauhan01@yahoo.co.in

Abstract

This study is focused to analyze the expression profile of CXCL8 and CXCL10 chemokines genes and to address the contribution of these chemokines in late phase of *M.leprae* infection in human skin lesion samples. In this study skin biopsy samples from leprosy patients (n=35) were collected including BL-LL=12, BB=14, BT=8 and healthy volunteers (n=3). All the biopsy samples were collected in RNALater for mRNA expression study as well as 10% buffered formalin (BF) for histopathologic analysis. Total RNA was isolated from collected samples and cDNA was prepared. Level of mRNA expression of CXCL8 and CXCL10 chemokine genes was measured via q-PCR and in-situ RT-PCR to locate the presence of CXCL8 and CXCL10 chemokines inside *M. leprae* infected tissue. The mRNA expression of CXCL8 was found relatively elevated in lepromatous (BL-LL) skin samples significantly as compared to BB category of leprosy; whereas in BT cases down-regulation was recorded. CXCL10 mRNA expression was found elevated in lepromatous (BL-LL) cases than other cases (BT, BB) of leprosy samples. In histopathological examination, 59.38% maximum infiltration was observed in BL-LL cases of leprosy and in-situ RT-PCR confirmed the presence of chemokines genes in tissue sections. Interestingly, chemokines CXCL-10 and CXCL-8 showed elevated expression in BL-LL category. The study advocated that CXCL10 and CXCL-8 possibly may have a role in lepromatous (BL-LL) form of leprosy.

Keywords: CXCL8; CXCL10; Leprosy; *M.leprae*; in-situ RT-PCR

Introduction

Leprosy, a granulomatous disease derived by *Mycobacterium leprae*, a very unique infectious agent generally affects the peripheral nerves and skin [1,2]. Several workers have investigated biomarkers, such as, chemokine, cytokine, and other receptors, including anti-PGL-1 antibody as well as cell markers across the leprosy spectrum and reaction [3-5]. Several previous reports concentrate on the role of chemokine IL-8, which magnetize neutrophil cells, T-lymphocytes, and probably monocytes [6] leading phagocytes of *M.tuberculosis* [7].

Chemokines are effective chemo-attractants for specific leukocytes and contribute in various chronic inflammatory processes [8-9]. The chemokine bio-molecules are minute, mostly secreted, peptides about 8-14 kDa, that are essential for the migration of immune cells and other cell mediators on the place of infection.

They also required for various essential cell functions, some of which overlap among their immunological reactions. The chemokines MIP-1α and MCP-1 were reported as elevated in pleural endothelial cells, monocytes and macrophage in patients suffering with active TB but RENTES was not reported as elevated [9,10], although IL-8 chemokine reported as up-regulated in macrophages [9].

Increased expression levels of TNF-α contained by leprosy patients suggested a role in favor of TNF-R2 in the regulation of this cytokine at some point during treatment [11]. In a follow-up study, levels of cytokines IFN-γ, TNF-α and IL-6R were reported as elevated in

Erythema Nodosum Leprosum (ENL) as compare to non ENL and declined after corticosteroid treatment of ENL in serum [12].

In a previous study, C-C chemokines were found elevated in plasma level in paucibacillary leprosy patients [13] and decreased after multi-drug therapy (MDT) in leprosy infected patients [14]. Not only CC chemokine, expression level of CXC chemokine such as CXCL-8,CXC-9,CXC-L10 also were found decreased after anti-tubercle treatment in tuberculosis infection [15] and CXCL10 reduced in leprosy skin lesion after treatment in those patients who did not have type 1 reaction (T1R) [16].

Previously, it has been reported that IL-8, MCP-1 and RANTES were found elevated in BT leprosy in reversal reaction and involved in recruiting monocytes and lymphocytes [17]. IP-10 (CXCL10) was found up-regulated in epidermis and dermal tuberculoid granulomatous lesions where as it was not elevated in lepromatous lesions [18].

Another study has claimed that CXCL10 and IL6 were elevated in plasma and could be potential biomarkers for T1R [19]. However, due to lack of information about the expression level of CXC chemokines such as CXCL8 and CXCL10 in untreated leprosy patients across the spectrum, this study was initiated.

This study may help to provide an opportunity to investigate the mRNA expression level of CXCL8 and CXCL10 chemokines in human leprosy skin lesions in untreated leprosy patients.

Material and Methods

Ethics

This study was initiated after ethical approval by the institutional Human Ethical Committee (HEC) at National JALMA Institute for Leprosy & OMD, Tajganj, Agra, UP, India.

Collection of leprosy specimens

All the skin biopsy samples were obtained from untreated leprosy patients attending the OPD, NJIL&OMD and control samples (skin samples from accidental cases) were collected from SN Medical College, Agra, UP, India. Cases were clinically categorized according to Indian Association of Leprologists (1982) in to borderline tuberculoid leprosy (BT, n=8), borderline category (BB, n=14), and lepromatous category (BL-LL, n=12).

Punch biopsies (in 4 - 6 mm diameter) were taken under local anaesthetic using 2% xylocaine (2 ml) injected intradermally, by inserting the needle into the centre of the lesion and driven inwards in stages, local anesthetic being injected at each stage. Scalpel biopsies (weighing approximately 100 mg) from active lesions of leprosy patients were collected under aseptic conditions using local anesthesia in RNALater solution (RNA Stabilization solution; Sigma, USA) from OPD, NJIL&OMD, Agra and immediately stored at -70°C.

Histopathological study

Punch biopsies, 4-6 mm in diameter were collected in RNALater to preserve RNA for mRNA expression study and same biopsy were collected in 10% buffered formalin (BF) for histopathological sectioning and analysis. Normal skin biopsy samples from vigorous volunteers (n=3) were also received following same procedure.

Eighteen to twenty-four hours afterward, all the tissues samples were embedded for paraffin sectioning and (5 μm) sections were sliced by microtome (Lieca, Germany) at room temperature and wrinkle free sections were immediately fixed onto 2% (3-aminopropyl) triethoxy-silane (Sigma, USA) coated slides. The section fixed slides were dewaxed in xylene, rehydrated in alcohol, stained with H&E and Fite-Farraco staining [20]. The stained slides were observed and analyzed under microscope (Olympus BX51, Japan).

RNA isolation and cDNA synthesis

RNA isolated from collected skin biopsy samples of control and leprosy infected patients (collected in RNALater) by using commercially available Tri-Reagent (Sigma, USA) subsequently, RT-PCR was performed with thermal cycler machine using cDNA synthesis kit (Fermantas, Germany) according to the manufacturer's instructions.

Standardization of cycling conditions for target chemokine genes by semi quantitative RT-PCR

Before applying the cDNA samples for expression analysis by Real Time PCR, few DNA (n=8) samples were subjected for optimization of cycling conditions for each target chemokine primers sets CXCL-8 (Forward primer-5'-TCTGCAGCTCTGTGTGAAGG- 3', Reverse primer-5'-TAATTTCTGTGTTGGCGCAG-3') and CXCL-10 (Forward primer- 5'-TCAGCACCATGAATCAAACTG-3', Reverse primer- 5'-CCTCTGGTTTTAAGGAGATCT-3') and GAPDH (Forward primer 5'-GGCCTCCAAGGAGTAAGACC-3', Reverse primer 5'-CTGTGAGGAGGGGAGATTCA-3') designed by Primer-3 software (www.frodo.ei.mit.edu/primer3).The PCR was executed in 25 μl volume using 2.5 U/μl Taq DNA polymerase (Fermantas), 25 mM MgCl2, 10X Taq DNA polymerase buffer, 2 mM each dNTP and each 4 μM of forward and reverse primers and 500 ng of cDNA, for 35 cycles with a profile 94°C for 4s', 94°C for 45s', 60°C for 45s', 72°C for 45s' and 72°C for 5s'. The PCR products were then analyzed on 1.2% RNase free TBE- agarose gel and revealed an expected size of band without any primer dimers and non-specific amplification. The chemokine check was not found to be compromised by DNA contamination.

Real-time PCR

Approximately 5 μg of total isolated RNA from all collected skin biopsies were reverse transcribed using first strand cDNA synthesis kit (Fermantas, Germany). The prepared cDNA, was then used for amplification with primers sets for human chemokines CXCL-8 (IL-8), CXCL-10 (IP-10) with GAPDH (as mentioned above) using SYBR Green-I PCR kit using Light Cycler 480 (Roche). The fold induction produced in favor of mRNA expression was determined from Ct (threshold cycle) values normalized for GAPDH expression and then from control.

In-situ RT-PCR

Paraffin processed and embedded sections (5 μm) were used to execute in-situ RT-PCR on the silane coated slides using Anti-Digoxigenin-AP Fab fragment (Roche, Germany) by two step RT-PCR kit (Bangalore GeNei, India) as per manufacturer's instructions with primer sets of human chemokines CXCL-8 and CXCL-10 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Tissue sections from the above step were used to perform in-situ RT-PCR in a two step procedure.

The RT reaction was performed at 42°C for 1 hour using AMV RT-PCR kit (Genei, India) as per manufacturer's instructions. The amplification reaction was performed for CXCL10, CXCL-8 along with GAPDH genes with specific primers mentioned above by incorporating with the Dig labeled dTTP, using PCR dig labeling mix (Roche, Germany) and fast Taq DNA polymerase (Roche, Germany) under thermo-cycling conditions of denaturation at 94°C for 2 min, then 94°C for 30s, 65°C for 30s, 69°C for 90s for 30 cycles using PTC-200 thermo-cycler machine (MJ Research, USA). Subsequent to amplification, the sections were refixed in 4% para-formaldehyde, treated with blocking buffer, further allowed for incubation with Anti-Digoxigenin-AP Fab fragment (Roche, Germany) for 1 hour at 37°C. The color reaction was developed using NBT/BCIP (Roche, Germany), followed by counterstaining with using heamatoxylin and the slides were observed under microscope (BX51 Olympus, Japan) after mounting with DPX.

Results

Histopathological analysis of leprosy skin lesions

Histopathological analysis of leprosy skin lesions was done using Hematoxylin and Eosin staining as well as Fite Faraco staining and the findings are depicted in Table 2. Infiltration (mononuclear cells) was found in BT, BB and BL-LL case of leprosy with mean 32.50%, 34.13% and 59.50%, respectively. Granulomatous lesions were observed in BT, BB and BL-LL case of leprosy (Table 2, Figures 1A-1C). 28.57% of BB

and 91.66% of BL-LL case of leprosy patients were found AFB positive (Figure 1D), except BT (0/8), no AFB were found in BT case of leprosy.
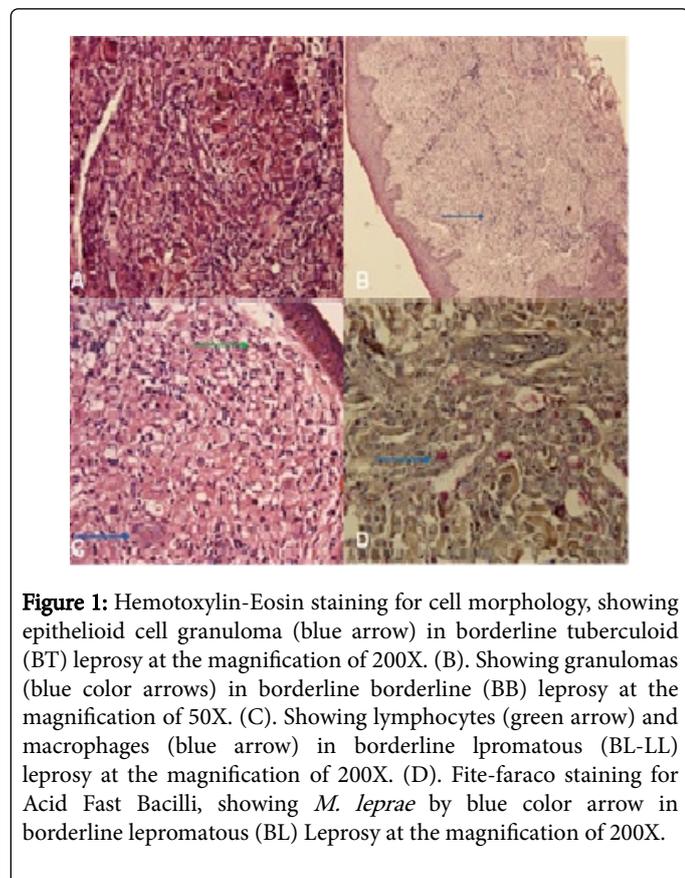
Figure 1: Hemotoxylin-Eosin staining for cell morphology, showing epithelioid cell granuloma (blue arrow) in borderline tuberculoid (BT) leprosy at the magnification of 200X. (B). Showing granulomas (blue color arrows) in borderline borderline (BB) leprosy at the magnification of 50X. (C). Showing lymphocytes (green arrow) and macrophages (blue arrow) in borderline lpromatous (BL-LL) leprosy at the magnification of 200X. (D). Fite-faraco staining for Acid Fast Bacilli, showing *M. leprae* by blue color arrow in borderline lepromatous (BL) Leprosy at the magnification of 200X.

Expression profile of CXC chemokine gene in leprosy skin lesions by Real Time RT- PCR

| S.No.	Type of Leprosy	Expression level of chemokine genes (mean log fold)	
		CXCL8	CXCL10
1	BT(n=8)	-0.54 ± 0.46	0.96 ± 0.29
2	BB(n=14)	0.97 ± 0.15	1.8 ± 0.41
3	BL-LL(n=12)	1.46 ± 0.13	2.18 ± 0.48

Table 1: CXCL8 and CXCL10 transcript level in leprosy skin lesions, significantly increased ($p < 0.05$) in BL-LL than BT cases of leprosy. The generated data was quantified by qPCR (in 100μg of total RNA) and normalized to GAPDH values. The results are presented as the Mean log fold ±SE.

CXCL10 mRNA was detected in extracted RNA, from all biopsy specimens (100 mg) from screened leprosy patients. However, the mean fold changes were 0.96, 1.8 log fold ($p > 0.05$) in biopsy samples from BT and BB patients of leprosy, respectively. Moreover, CXCL10 was found significantly up-regulated ($p < 0.05$) with 2.18 log fold expression in BL-LL case of leprosy (Table 1). But CXCL8 chemokine gene was found down regulated (-0.54 mean log fold) in skin lesions of leprosy BT patients. However, the CXCL8 chemokine mRNA were found significantly up-regulated (0.97 mean log fold, 1.46 log fold, $p < 0.05$) in skin lesions of BB and BL-LL leprosy patients relative to the BT cases of leprosy (Table 1).

In-situ expression of CXCL8 and CXCL10 mRNA

In-situ RT-PCR of leprosy skin lesions (positive in real-Time PCR) was performed to confirm the expression of CXCL8 and CXCL10 genes. The tissue sections fixed in 10% buffered formalin and were permeabilized by a thermo-cycling method. The cellular DNA treated with DNAse I then tissue sections were taken under reverse transcription PCR procedure in which biotin labeled dTTPs and same chemokine primers were used. Using in-situ RT-PCR expression of CXCL8 gene was observed in BL-LL as well as BB case of leprosy while no amplification signals was observed in BT type of leprosy patient except one out of eight samples. Out of total cases, in this study 91.66% (11/12), 85.71% (12/14) and 12.5% (1/8) case were found positive for CXCL8 in BL-LL, BB, and BT case of leprosy respectively (Figure 2D, Table 2).
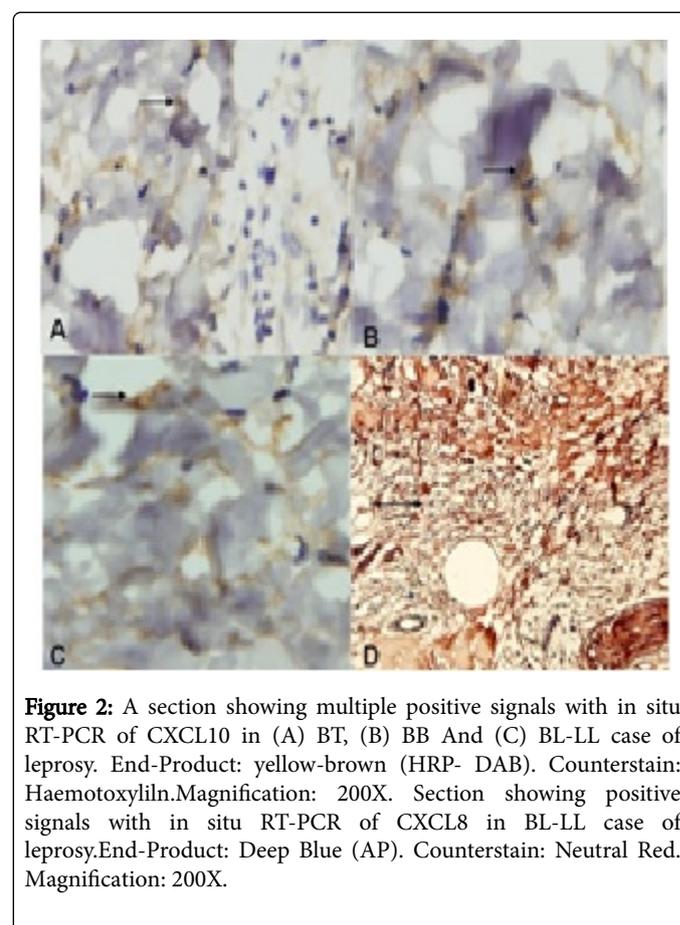
Figure 2: A section showing multiple positive signals with in situ RT-PCR of CXCL10 in (A) BT, (B) BB And (C) BL-LL case of leprosy. End-Product: yellow-brown (HRP- DAB). Counterstain: Haemotoxyliln.Magnification: 200X. Section showing positive signals with in situ RT-PCR of CXCL8 in BL-LL case of leprosy.End-Product: Deep Blue (AP). Counterstain: Neutral Red. Magnification: 200X.

CXCL10 mRNA was studied by in-situ RT-PCR in the sections with gene specific primers, amplification of signals were detected with steptavidin-Horse Radish Peroxidase (SA -HRP). It was observed that the CXCL10 chemokine gene was found expressed in lepromatous leprosy (BL-LL), borderline (BB) as well as in BT case of leprosy with 83.33% (n=10/12), 78.57% (n=11/14) and 62.5% (n=5/8) positivity, respectively (Figures 2A-2C and Table 2).

In summary, it was observed that the CXCL8 chemokine gene and CXCL10 chemokine gene were expressed in 24/34 and 26/34 cases respectively with positivity being seen more in the multibacillary (BL-LL, BB) group (Table 2) suggesting a possible role of these chemokines in the accumulation of granulomatous infiltrates in this form of the disease.

S.No.	Case Type	Mean of Infiltration (%)	AFB Positive case	Case +ve for CXCssL8	Case +ve for CXCL10
1	BT(n=8)	32.5	0	1 (12.5%)	5 (62.5%)
2	BB(n=14)	34.13	8 (57.14%)	12 (85.71%)	11 (78.57%)
3	BL-LL(n=12)	59.5	12 (100%)	11 (91.66%)	10 (83.33%)
4	Healthy (n=3)	-	-	-	-

Table 2: Histopathological observation and detection of transcription of chemokines of CXC family at the site of infection by in-situ RT-PCR using gene specific primers.

Discussion

The presence or expression of CXCL8 and CXCL10 chemokines were confirmed in leprosy patients. In this study, we found expression in chemokines CXCL8 and CXCL10 in skin lesions across the leprosy spectrum. It has been reported that CXCL8 play important role in monocytes and lymphocytes recruitment in leprosy lesions. Monocytes infected by *Mycobacterium tuberculosis*, revealed the in-vitro expression of CXCL8; however, macrophages may be a possible source of CXCL8 in leprosy lesions. Adding together, stimulated T lymphocytes may possibly also express CXCL8 as they have been revealed in in-vitro condition [21]. Our findings support the earlier suggestion that CXCL8 may take part in a positive role in the vibrant direction of immune cell invasion in leprosy lesions [17].

In addition, the outcomes from the study signify a correlation between the elevation of CXCL8 and CXCL10 in leprosy. BL-LL showed higher 59.50% infiltration as compared to BB (34%) and BT (32.50%) and granulomatous structure containing mononuclear cells (Table 2 and Figure 1). The CXCL10 mRNA expression in BB and BL-LL leprosy skin lesions indicate that CXCL10 may direct the infiltrating cells at the site of inflammation. Previously it has been reported that CXCL10is associated with T1R (type 1 reaction) in BT patients [19]. The strong relationship among elevated CXCL10 and clinical T1R suggest that CXCL10 chemokine may be helpful as a marker to serve in the diagnosis for leprosy as well as these chemokines may also useful in other mycobacterial diseases [16,22]. The previous published reports, exposed the differential expression of cytokine IFN-γ have frequently, but not consistently, been linked with TR1 [23]. Since, IFN-γ cytokine assumed as inducer of CXCL10, additionally, it is feasible that IFN-γ transcripts are produce early on in the reaction and then decline quickly, whereas CXCL10 chemokine expression delayed and for a longer time remains elevated. These findings showing the elevated expression of CXCL10 indicate that IFN-γ may have role in production of CXCL10 in leprosy skin lesions [18]. Whereas, Scollard et al. [16] observed association between elevation of IFN-γ and expression of CXCL10 in skin lesions but the sample size is too low to significance.

However, enhanced production of CXC chemokine is a well-documented consequence of infection among other mycobacterial diseases [24] and the expression of CXCL10 is associated with TR1 in leprosy sufferers. In the study, it was evaluated the presence of CXC chemokines (CXCL8 and CXCL10) in impure leprosy skin lesions. We observed that only CXCL8 and CXCL10 chemokines were found expressed within leprosy skin lesions. The mRNA expression of CXCL10 chemokine observed higher than CXCL8 in advanced stage of leprosy. Thus, CXCL8 and CXCL10 chemokine may have a role in leprosy skin lesions across the spectrum (BT, BB, BL-LL), whereas other chemokines may need to investigate, which may involve in disease spectrum of leprosy. Due to limitation of reagents and chemicals, we were unable to investigate the other chemokines and cytokine.

In sum, it was observed that CXCL10 and CXCL8 chemokines were found to be expressed in BT, BB, BL-LL and BB, BL-LL leprosy skin lesions, and increase in advance stages of leprosy, but their levels varied. Thus these results indicate that CXCL8 and CXCL10 may have a role in disease pathology across the spectrum of leprosy. In situ RT-PCR is useful to demonstrate transcripts in the cells and capable to detect mRNA expression inside the Mycobacteria infected tissue.

Acknowledgements

Conflict of Interest
None

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