Cortisol and proinflammatory cytokine profiles in type 1 (reversal) reactions of leprosy

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Purpose: Cortisol levels in the circulation and at the sites of peripheral inflammation regulate type 1 (Reversal) reactions in leprosy skin to delayed type hypersensitivity reactions (DTH). In this study we determine the extent to which the differential mRNA expression of genes encoding cortisone–cortisol shuttle enzymes (11 β hydroxysteroid dehydrogenase I & II (11 β HSD I & II)), circulatory levels of pro-inflammatory cytokines (IL-6, IL-7, IP-10, IL-17F, IL-23, TNF-α, IL-1β, PDGF BB and CRP) and cortisol are associated with development of type 1 reactions in leprosy.

Methods: Urine, blood and incisional skin biopsy samples from site of lesions were collected from 49 newly diagnosed untreated leprosy cases in T1R and 51 cases not in reaction (NR). mRNA expression levels of genes encoding 11 β HSD I & II in skin biopsy samples were determined by realtime PCR. Cortisol levels from the lesional skin biopsies, serum and urine samples and serum proinflammatory cytokine levels were measured using ELISA.

Results: The mean expression ratios of 11 β HSD I & II are significantly lower in leprosy cases with T1R when compared to the NR leprosy cases. Cortisol levels in lesional skin biopsies and in urine are significantly lower (p = 0.001) in leprosy cases with T1R. Serum cytokine levels of IP-10, IL-17F, IL-IL-6 and TNF-α are significantly higher (p < 0.05) in leprosy cases with T1R when compared to the NR leprosy cases.

Conclusion: Our study indicated an association of urinary and lesional skin cortisol levels with the manifestation of T1R in leprosy. IP-10, IL-17F, IL-6 and TNF-α can be potential prognostic serological markers and gene expression markers for early detection of type 1 reactions in leprosy.

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1. Introduction

Leprosy is a chronic infectious disease caused by an obli-
gate intracellular bacillus called Mycobacterium leprae (M. leprae). Disease progress through an immunological spectrum with two distinct poles, cell mediated immunity (CMI) preponderated self-limiting tuberculoid pole which gradients through three inter-
mediary borderlines forms towards the lepromatous pole which is manifested by a decrease in M. leprae specific CMI and concomi-
tant increase in humoral immunity [1]. Patients who are mostly in the borderline forms of the disease experience exacerbations of the immune system, which are often manifested as delayed type hypersensitivity reactions called type 1 reactions (T1R) in Leprosy [2].

The T1R is characterized by the inflammation in the skin and in the nerves or in both. Apart from its predominance in the borderline forms of the disease, a part of the patients in the polar forms also manifest these reactions. Clinical characteristics include oedema of the hands, face and feet along with erythema-
matous and/or oedematous skin lesions with without ulceration [3]. Systemic symptoms are unusual but acute inflammation of the peripheral nerves occur leading to nerve function impairment (NFI) which when not treated rapidly and adequately will lead to per-
manent loss of nerve function which is often characterized by the development of deformities of various grade [4]. These reactions occur before, during and after the multi drug therapy (MDT) and approximately 30% of the diagnosed leprosy patients experience these reactions [2]. These reactions are treated with oral corticos-
teroids and approximately 60% of the patients recover from the NFI [3].

Several attempts have been made to understand the mechanism of T1R and it has been shown that there is spontaneous rise of in situ Th1 type of immunity with infiltration of CD4+ T cells in

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skin and nerve lesions with increase of tumour necrosis factor-α (TNF-α) and interferon-γ (IFN γ) in the local miel in TIR [5]. A recent study carried out by Sousa et al. [6] claimed that M. leprae positivity by PCR in single lesion (>5 cm) Paucibacillary (PB) cases (>40 years) indicated predictors of type 1 reaction. Earlier studies indicated that during both the type1 and type 2 reactions in most of the cases, there was higher signals of IFN γ in the skin than non reactive patients [7]. However, recent studies reveal both these reactions did not show any difference in their IFN γ levels [8]. A longitudinal cohort multi-centric study using the parameters based on temperature and nerve functions failed to predict TIR [9]. The CMI up-regulation in TIR seems to be spontaneous in nature and is a major cause of neuritis often leading to nerve damage. However, till today the mechanism underlying for the sudden upsurge in CMI is largely unknown. The diagnosis of TIR is still based on clinical parameters because histopathological changes mostly fail to differentiate between relapse and reactivation. A recent cohort study could not also associate the levels of TNF-α – an inflammation molecule and antibody levels against S100, LAM, PGL-1 and Ceramide with TIR [10]. However, an earlier study of Stefani et al. [11] and a recent finding by Scollard et al. [12] indicated a significant rise in CXC ligand 10CXCL10 (IP10) in tissues and serum during reaction but whether this will be able to predict a patient going into reaction is not known.

Circulatory cortisol reaches the tissues in inflammation however the local skin concentrations are regulated by inter-conversion of active cortisol to inactive cortisone and vice versa. The enzymes involved in these conversions are called Cortisol-cortisone shuttle enzymes namely 11β-hydroxysteroid dehydrogenase (11B-HSD) I which mediates NADP (H) dependent conversion of inactive cortisone to active cortisol and 11β-hydroxysteroid dehydrogenase II which acts exclusively as an oxidase converting cortisol to cortisone with NAD as its co-factor.

Cortisol regulates localized inflammation in the delayed type hypersensitivity reactions apart from its systemic role as a stress hormone in the control of carbohydrate, protein and fat metabolism. Glucocorticoids such as prednisolone are widely used in the control of peripheral inflammations in Leprosy and many other autoimmune diseases. Glucocorticoid (GC) such as cortisol acts as an immunosuppressant by regulating several transcription factors including activating protein (AP)-1, NF-κB, and nuclear factor of activated T-cells (NFAT) [13]. GCs inhibit Th1 cytokines and promote a shift towards Th2 differentiation. Cortisol regulates the circulatory and expression patterns of IFN γ [14], TNF-α, interleukin-1β (IL1β), C-reactive protein (CRP) [15] and interleukin 6 (IL6) [16]. It has been demonstrated in the Crohn’s disease and ulcerative colitis models that serum and excretory levels of cortisone significantly increase in the site of inflammation [17]. Studies on cortisol metabolism in cases with TIR reveal impaired localized regulation of cortisol, which is mediated by cortisol–cortisone shuttle enzymes may lead to the development of erythematous skin lesions [18].

Understanding the cortisol regulatory mechanisms underlying manifestation of these type 1 skin reactions in localized skin lesions may aid in identifying methods for early diagnosis and early treatment intervention to prevent nerve damage and consequent deformities. In this study, we tested the hypothesis that the acute inflammations during the type 1 reactions are associated with disruption of the cortisol–cortisone shuttle enzymes [18].

We examined the mRNA expression profiles of cortisol regulating enzymes in the skin lesions of newly diagnosed untreated leprosy cases in type 1 reactions and also in cases without any reactions. Concomitantly, the cortisol levels in serum, urine and lesonal skin total protein isolates and serum levels of cytokines (IL-6, IL-7, IP-10, IL-17F, TNF-α, IL-1β, PDGF BB and CRP) were estimated to perform a cross-sectional association with cortisol regulation and with the development of TIR in leprosy.

2. Methods

2.1. Study subjects

A total of 49 leprosy cases in TIR and 51 leprosy cases without any reaction (NR)(controls) were enrolled in the study after taking informed consent for participation following the ethical standards and guidelines of the Indian Council of Medical Research and the host organization-The Leprosy Mission Trust India. All the study subjects were newly diagnosed untreated leprosy cases attending The Leprosy Mission Community Hospital, Shahdara, New Delhi, India. Care has been taken to exclude any cases with previous history of MDT treatment, defaulters and treated with hydrocortisone (prednisolone). Cases with HIV positive status and/or having dermatophytic infections that are known to elicit cortisol releases are excluded from the study. There is no significant variation (p > 0.05) observed in the age groups and gender distribution between the cases and controls (Table 1). All the cases were clinically examined by experienced dermatologists and the Ridley Joping (RJ) classification was determined from histopathological examination of biopsy specimens. Cases in TIR are clinically determined based on the manifestation of erythematous/oedematous skin lesions and oedema of the face, hands and feet and sometimes accompanied by neuritis. Nerve function impairment (NFI) was assessed in all the study subjects to identify the motor and sensory loss using voluntary muscle testing and Semmes–Weinstein monofilaments as described earlier [10].

2.2. Sample details

5 mm × 5 mm incisional skin biopsies at skin lesion, 3 ml of peripheral venous blood (collected between 8:00 AM and 10:00 AM owing to the diurnal changes in the serum cortisol levels) and 24 h urine samples were collected from each subject who were enrolled in the study. The biopsy samples were collected in RNA later in aseptic conditions by a clinician and were sent to Stanley Browne laboratory for RNA extraction and realtime PCR experiments. Serum was separated from the blood and 3 µl/ml of Protease Inhibitor Cocktail (Cat No.: PB340, Sigma–Aldrich Inc., USA) was added and stored at −20 °C freezer until further processing. Owing to the diurnal changes in the excretory levels of cortisol [19] in humans, 24 h total urine output was collected, aliquoted and stored in −20 °C freezer until cortisol ELISA was performed.

2.3. RNA extraction and real time PCR

2.3.1. RNA extraction

Aseptically, a 2 mm × 2 mm size skin tissues were cut from the current biopsy sample and minced/grinded thoroughly in laminar hood using manual glass homogenizer and/or Polytron (Kinematica PT 2100) in liquid phase using TRI-reagent (Sigma–Aldrich, Cat No.: T9424) according to the manufacturer’s instructions. Briefly, the homogenized tissue (in 1 ml TRI Reagent) was centrifuged at 12,000 × g for 10 min at 4 °C followed by phase separation with 200 µl of Chloroform. DNA was extracted from the upper aqueous phase using 500 µl of cold isopropanol, the pellet was washed with 75% ethanol and stored at −80 °C freezer or processed by drying the pellet followed by dissolution in DEPC treated water. The RNA suspensions are treated with DNase I (Cat No.: EN0521, Fermentas Inc.) according to the manufacturer’s instructions to remove any traces of genomic DNA contamination. The concentrations of the
RNAs samples were determined spectrophotometrically on a UV Spectrophotometer (Shimadzu Inc. Japan).

2.3.2. cDNA construction

cDNA was constructed from 1 μg of total RNA from each of the sample using Protoscript® M-MuLV First Strand cDNA Synthesis Kit (NEB #63005, New England Biolabs Inc). Briefly, 1 μg of total RNA was mixed with Random Primer mix and nuclease free water. RNA was then denatured at 70 °C for 5 min. This is followed by the addition of 1 x M-MuLV Reaction Mix containing buffer, Magnesium ions, dNTPs and 1 x M-MuLV Enzyme Mix containing 0.5 units/μl of Reverse Transcriptase and RNase Inhibitor. Temperature cycling conditions included 25 °C for 5 min, 45 °C for 1 h followed by inactivation of enzyme at 80 °C for 5 min.

2.3.3. Realtime PCR

cDNA corresponding to 11β HSD 1 and 11β HSD II transcripts are amplified on Rotor Gene-Q (Qiagen Inc. USA) realtime PCR machine using primers and reaction conditions as published earlier [18]. Briefly, 10 μl of Rotor-Gene SYBR Green PCR Master Mix (Qiagen, Cat No.: 204074), 200 nM concentration of each of the forward and reverse primers for 11β HSD 1 and 11β HSD II (Table 2), and 200 ng of cDNA (total of 20 μl reaction mix) are amplified in Rotor-Gene Q with cycling conditions as 95 °C for 10 min (initial denaturation and activation of enzyme) followed by 40 cycle of 95 °C for 10 s, annealing at 61 °C for 15 s for 11β HSD 1 (57 °C for 11β HSD II) and elongation at 72 °C for 20 s. The annealing temperatures for each of the gene targets were taken from the published work [18]. The fluorescence was acquired on green channel during the annealing step. A melting curve analysis was performed by heating the amplitcons from 65 °C to 95 °C with 1 °C/s rise in temperature at each step. The mRNA expression levels were normalized by using GAPDH (glyceraldehyde 3 phosphate dehydrogenase) mRNA as a house-keeping gene. The threshold fluorescence values were normalized to those of GAPDH values. The mRNA expression levels were calculated after determining the primer efficacy for all the targets using Pfaffl Method [20] by a standard curve with a 6 fold dilution of cDNA from 1 μg/reaction to 10 ng/reaction. Melting curve analysis was performed to determine the integrity of the amplification and to rule out primer–dimer formation.

The percentage efficiency of the primers from the standard graphs were determined to be in the order of 98% for GAPDH, 93% for 11 β HSD I, 91% for 11 β HSD II, 97% for IP-10, 98% for TNF-α, 97% for IL-17F and 91% for IL-23R. The fold difference in expression was calculated based on the below formula:

\[
\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_T}_{\text{Control}}}{(E_{\text{ref}})^{\Delta C_T}_{\text{Control}}}
\]

Individual expression ratios using the above formula were computed for cases in TIR and NR leprosy cases taking the average ratio of NR as the control value for all the cases. The statistical difference was calculated using non-parametric Mann–Whitney U test.

2.3.4. Realtime PCR for expression levels of cytokine coding genes in skin lesions

cDNA corresponding to IP-10, TNF-α IL-17F and IL-23R, transcripts was also amplified on Rotor Gene-Q (Qiagen Inc., USA) realtime PCR machine using primers and reaction conditions as published earlier [21–24]. Expression ratios were calculated for representative set of TIR (n = 30) and NR leprosy cases (n = 30) with reaction conditions and analysis as described in the previous section.

2.4. Total protein isolation from skin biopsy samples

As mentioned earlier under RNA extraction procedure, the phase separation using TRI reagent and chloroform separates the sample into three phases, the upper aqueous phase containing RNA, the middle interphase containing the DNA and the lower phenol–ethanol phase containing the proteins. Total protein was extracted using TRI-reagent following manufacturer’s instructions. Briefly, the phenol–ethanol phase was removed and 1.5 ml of isopropanol per 1 ml of TRI reagent was added and samples were allowed to stand at room temperature for 10 min followed by centrifugation at 12,000 × g for 10 min at 2–8 °C. The supernatant is discarded and pellet is washed three times with 75% ethanol in 20 min intervals. The pellet was then air dried and dissolved in 1% SDS (sodium dodecyl sulphate). The protein concentration was determined spectrophotometrically on a UV Spectrophotometer (Shimadzu Inc. Japan).

2.5. ELISA for cortisol

A competitive ELISA was performed with urine, serum (1:20 dilution) and skin total protein samples (1:10 dilution of 1 μg total protein) using Parameter Cortisol Assay Kits (Cat No.: KGE0008, R&D Systems Inc., USA). An inverse logarithmic curve was used with 7-fold dilution of cortisol standard and the test concentrations were plotted on the graph.

2.6. ELISA for cytokines and growth factors (IL-6, IL-7, IP-10, IL-17F, IL-23, TNF-α, IL-1β, PDGF BB, CRP)

A sandwich ELISA was performed for IL-6, IL-7, IP-10, IL-17F, IL-23, TNF-α, IL-1β, PDGF BB and CRP using ELISA kits (R&D Systems Inc. USA). Briefly the 96 well microtiter plates were coated with 100 μl/well working concentration of mouse anti-human antibodies for the above molecules and incubated overnight at room temperature. Followed by washing and blocking with a block buffer (400 μl/well) containing 1% BSA in PBS for 1 h, the plates were incubated with 100 μl/well of standards, controls and samples for appropriate wells and incubated for 2 h at room temperature. This is followed by the addition of 100 μl/well of detection antibody (biotinylated goat-anti-human antibody for above molecules) and incubation at room temperature for 2 h. The plates were then washed and incubated with 100 μl/well of...
of streptavidin conjugated to horseradish-peroxidase for 20 min in dark followed by 100 µl/well of substrate solution containing equal volumes of tetramethylbenzidine and hydrogen peroxide. The reaction was stopped and the plates were read at 450 nm on a Dynatech MR 2000 ELISA Plate Reader.

2.7. Statistical analysis of the data

The statistical analysis was performed using Graph-Pad Prism software (Version 6). The real-time data was analyzed on Rotor-Gene Q Series Software (Software Version 2.0.2). Cortisol and cytokine levels were analyzed using non-parametric Mann–Whitney U test. The correlations were performed using Spearman Rank correlation analysis. An association with p value less that 0.05 is considered as statistically significant.

3. Results

3.1. mRNA expression profiles of 11ß HSD I and 11ß HSD-II in the skin lesions

The fluorescence data, which was acquired on green channel was analyzed by using Rotor Gene Q Software (Qiagen Inc., USA). The fold difference in expression between the target (11ß HSD I & II) and the reference (GAPDH) genes were computed considering cases in T1R as sample group and cases without reaction as the control group. The mean mRNA expression ratios of 11ß HSD I/GAPDH are identified to be significantly lower in leprosy cases with T1R in comparison to the NR leprosy cases (T1R vs. NR, 1.507 vs. 4.674, p < 0.05) (Fig. 1). Similar observations were also made in 11ß HSD II where, a statistically significant difference was observed in the mean mRNA expression ratios of 11ß HSD II/GAPDH between both the study groups (T1R vs. NR, 0.944 vs. 2.040, p < 0.05) (Fig. 2).

3.2. mRNA expression profiles of IP-10, TNF-α, IL17F and IL-23R in skin lesions

The mean mRNA expression ratios of IP-10/GAPDH (T1R vs. NR, 43.08 vs. 9.94, p < 0.05) (Fig. 12), TNF-α/GAPDH (T1R vs. NR, 19.68 vs. 8.047, p < 0.05) (Fig. 13), IL-17F (T1R vs. NR, 7.17 vs. 1.43, p < 0.05) (Fig. 14) in the skin lesions are identified to be significantly higher in leprosy cases with T1R in comparison to NR leprosy cases. However, comparative analysis of mRNA expression levels of IL-23R (T1R vs. NR, 2.76 vs. 2.06, p < 0.05) (Fig. 15) revealed no statistically significant difference across the study groups.

3.3. Localized and systemic cortisol levels

Estimation of the tissue cortisol levels revealed that the mean cortisol levels in the skin lesions of leprosy cases with T1R are significantly lower in comparison to those in the skin lesions of the NR leprosy cases. The cortisol levels are estimated in 1 mg of total protein isolated from each biopsy sample (T1R vs. NR, 2.760 ng/ml vs. 3.335 ng/ml, p < 0.05) (Fig. 3). Owing to the diurnal changes in the excretory levels of cortisol and presence of high levels of cortisol in the urine samples collected during the morning hours [25], a 24 h urine sample is collected and 1 ml aliquot is used for the estimation of total cortisol excreted in 24 h. The cortisol levels in the urine are identified to be significantly lower in cases with T1R in comparison to the NR cases (110.0 ng/ml vs. 212.7 ng/ml, p < 0.05) (Fig. 4).

However, the serum cortisol levels were also estimated which revealed that the differences between both the study groups are not statistically significant. (T1R vs. NR, 114.6 pg/ml vs. 108.8 pg/ml, p < 0.05) (Fig. 5).

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**Table 2**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Annealing temperature</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'S'-TTGGTATCTGGAGAAGCACA-3'</td>
<td>60°C</td>
<td>270bp</td>
</tr>
<tr>
<td>11ß HSD I</td>
<td>5'S'-TGCAACACAAACACCACGAGG-3'</td>
<td>61°C</td>
<td>150bp</td>
</tr>
<tr>
<td>11ß HSD II</td>
<td>3'S'-TCGAAAGCTGTCGACGTGA-5'</td>
<td>57°C</td>
<td>129bp</td>
</tr>
</tbody>
</table>

**Fig. 1.** Comparative analysis of mRNA expression ratios of 11ß HSD I/GAPDH in lesional skin biopsy samples between the leprosy cases in T1R (n = 49) and NR leprosy cases (n = 51) using realtime PCR.

**Fig. 2.** Comparative analysis of mRNA expression ratios of 11ß HSD II/GAPDH in lesional skin biopsy samples between the leprosy cases in T1R (n = 49) and NR leprosy cases (n = 51) using realtime PCR.
Fig. 3. Comparative analysis of cortisol levels in lesional skin biopsies samples (1 mg of total protein isolate) between the leprosy cases in T1R (n = 49) and NR leprosy cases (n = 51) using ELISA.

Fig. 4. Comparative analysis of cortisol levels in 24 h urine samples between the leprosy cases in T1R (n = 49) and NR leprosy cases (n = 51) using ELISA.

Fig. 5. Comparative analysis of cortisol levels in serum samples between the leprosy cases in T1R (n = 49) and NR leprosy cases (n = 51) using ELISA.

Fig. 6. Comparative analysis of inducible protein 10 (IP-10) levels in serum samples between the leprosy cases in T1R (n = 49) and NR leprosy cases (n = 51) using ELISA.

Fig. 7. Comparative analysis of interleukin 17F levels in serum samples between the leprosy cases in T1R (n = 49) and NR leprosy cases (n = 51) using ELISA.

3.4. Serum cytokine levels

In addition to cortisol, circulatory profiles of various proinflammatory cytokines were also studied in order to understand their cross-sectional association with manifestation of T1R in leprosy. Serum levels of IL-6, IL-7, IP-10, IL-17F, IL-23, TNF-α, IL-1β, PDGF BB and CRP were estimated using ELISA. We observed a statistically significant higher levels of IP-10 (T1R vs. NR, 665.7 pg/ml vs. 422.0 pg/ml, p < 0.05) (Fig. 6), IL-17F (T1R vs. NR, 521.2 pg/ml vs. 428.8 pg/ml, p < 0.05) (Fig. 7), IL-6 (T1R vs. NR, 17.49 pg/ml vs. 9.67 pg/ml, p = 0.03) (Fig. 8) and TNF-α (T1R vs. NR, 104.3 pg/ml vs. 23.55 pg/ml, p < 0.05) (Fig. 9) in leprosy cases with T1R in comparison to NR leprosy cases. The observations are analyzed in a cross-sectional design involving only one time point of sample collection i.e. at the time of recruitment and before the initiation of MDT/Steroid treatment.

However, analysis of the serum levels of IL-7 (T1R vs. NR, 13.58 pg/ml vs. 11.56 pg/ml, p > 0.05), IL-23 (T1R vs. NR, 48.73 pg/ml vs. 46.93 pg/ml, p > 0.05), IL-1β (T1R vs. NR, 31.17 pg/ml vs.
Fig. 8. Comparative analysis of interleukin 6 levels in serum samples between the leprosy cases in TIR (n = 49) and NR leprosy cases (n = 51) using ELISA.

![Graph showing interleukin 6 levels](image)

### Study Groups

104.9 pg/ml, \( p > 0.05 \), PDGF-BB (TIR vs. NR, 294.4 pg/ml vs. 454.5 pg/ml, \( p > 0.05 \)) & CRP (TIR vs. NR, 6904 pg/ml vs. 6702 pg/ml, \( p > 0.05 \)) revealed that the differences across the study groups are not statistically significant.

3.5. **Correlations between serum cortisol and IP-10 and cortisol and TNF-α**

Spearman Rank correlation analysis was performed between the serum levels of cortisol and IP-10 and between cortisol and TNF-α. Observations indicated a statistically significant negative correlation between serum levels of cortisol and IP-10 with a correlation coefficient \( r \) value of \(-0.2542\) (\( p < 0.05 \)) (Fig. 10) and between serum levels of cortisol and TNF-α with a correlation coefficient \( r \) value of \(-0.1854\) (\( p < 0.05 \)) (Fig. 11). These correlations were performed within the entire study group involving both the cases in TIR and NR leprosy cases.

![Graph showing cortisol and IP-10 correlation](image)

**Fig. 10.** Spearman rank correlation coefficient of serum cortisol and serum IP-10 levels across the entire study group (leprosy cases in TIR + NR leprosy cases) (\( n = 100 \)).

![Graph showing cortisol and TNF-α correlation](image)

**Fig. 11.** Spearman rank correlation coefficient of serum cortisol and serum TNF-α levels across the entire study group (leprosy cases in TIR + NR leprosy cases) (\( n = 100 \)).

### 4. Discussion

Cortisol levels in circulation and in the periphery regulate an array of proinflammatory cytokines, which in turn control the localized inflammations [26]. *M. leprae* appears to have a specific tropism for peripheral nerves, where it resides primarily in the myelinating Schwann cells and in the macrophages that have migrated through the blood-nerve barrier into the endoneural...
The presence of mycobacterial antigens in leprosy exposes the nerves to damage during the acute inflammatory episodes. Cortisol has anti-inflammatory effects and the local concentration of cortisol in any cell or tissue is not dependent only on the concentration reaching that tissue from the circulation but also on enzymes namely 11βHSD 1 and 11βHSD II, which are collectively referred to as the cortisol-cortisone shuttle enzymes, present at the site that regulate the local concentrations to suit the needs of the tissue. The proinflammatory cytokines TNF-α and IL-1β are known to upregulate reductase activity of the shuttle enzyme converting inactive cortisone to active cortisol [28]. Any defect in these activities is likely to affect the inherent anti-inflammatory mechanism severely making patients either prone to reactions or making them non-responsive to steroid treatment.

The inflammation in leprosy lesions is fairly unstable and most studies [12,29] focus on the immunological and cytokine networks operating in the leprosy lesions however not many studies were pursued on understanding the localized cortisol metabolism in type 1 reactional skin lesions [18,30].

The cellular and molecular mechanisms involving manifestation of T1R in the skin lesions has been studied in detail. Histopathological findings reveal that the T1R skin lesions show all characteristics of a delayed type hypersensitivity reactions [31]. Presence of extracellular oedema, proliferation of the fibroblasts and change in the cellular composition in and around the epitheloid cell granuloma are some of the major characteristics of T1R skin lesions which is mainly due to the influx of Th1 class CD4 lymphocytes [32,33]. Various studies have demonstrated increased expression of proinflammatory cytokines – TNF-α, IFN γ, IL-6, IL-12, TGF β and IL-10 in reactional skin lesions along with the evidence of macrophage activation [8,34]. A significant decrease in expression of the lesional cytokine mRNAs [35] has been noted with the corticosteroid therapy and cortisol-cortisone shuttle enzymes themselves regulate the tissue specific proinflammatory cytokine expression profiles [36]. Based on these observations, we hypothesized possible disruption of the cortisol-cortisone shuttle system as one of the major causes for the failure of endogenous mechanism involved in the control of peripheral inflammations in T1R.

It has been demonstrated in the psoriasis model that localized cortisol levels in the skin inflammations reduce to a greater extent leading to the development of erythematous and oedematous skin aberrations [37]. Our results indicate a similar observation where the localized tissue cortisol levels decreased in type 1 reactional skin lesions when compared to those that are not in reaction. Stress signals activate the hypothalamus–pituitary–adrenal (HPA) axis and the sympathetic nervous system. Elements derived from these systems (e.g., cortisol, catecholamine and neuropeptides) can influence the immune system and possible disease states. Cutaneous innate and adaptive immune responses are largely affected by stress regulatory molecules and inflammatory episodes cascade a variety of stimulatory molecules that activate the HPA axis and regulate the circulatory and excretory levels of cortisol [38]. It has been shown in the posttraumatic stress disorder and psoriasis model that urinary cortisol metabolites reduce dramatically after provoked by various inflammatory stressors [39]. We also observed significantly lower levels of cortisol in 24 h urine samples in cases with T1R when compared to that of NR.

We observed significantly lower levels of urinary cortisol in cases with T1R however, this observation is not similar in the serum levels of cortisol (no significant difference across the study groups). One of the possible reasons for this observation is the diurnal changes in the circulatory and excretory profiles of cortisol due to which the urinary free cortisol estimations where performed in 24 h urine samples. However, plasma cortisol also exhibits diurnal variance [40] with elevated levels in the morning hours, which gradually decline through evening till midnight. One of the limitations of space [27].

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**Fig. 13.** Comparative analysis of tumour necrosis factor α (TNF-α)/GAPDH mRNA expression ratios between the leprosy cases in T1R (n = 30) and NR leprosy cases (n = 30) using realtime PCR.

**Fig. 14.** Comparative analysis of interleukin 17F (IL-17F)/GAPDH mRNA expression ratios between the leprosy cases in T1R (n = 30) and NR leprosy cases (n = 30) using realtime PCR.

**Fig. 15.** Comparative analysis of interleukin 23R (IL-23R)/GAPDH mRNA expression ratios between the leprosy cases in T1R (n = 30) and NR leprosy cases (n = 30) using realtime PCR.
this study is that a uniform time point was not maintained in the collection of plasma samples due to the patients visiting the OPD of the hospital at different times in a day. Efforts have been made to confine the sample collection times to a 3 h time slot in the forenoon but not all cases were collected during the early morning hours.

Proinflammatory cytokines play a vital role in the regulation of type 1 reactions in leprosy. IP-10 is induced by IFN-γ in several cell types in response to various inflammations. It was observed in asthmatic airway inflammation model that the pro-inflammatory cytokines upsurge with a concomitant fall in the anti-inflammatory cytokines and cortisol [41]. We observed a significant increase in IP-10 in type 1 reactions of leprosy and a significant negative correlation with serum cortisol levels. Similar findings were observed with TNF-α in cases with T1R which was earlier demonstrated in the osteoblast model where modulation of 11 β HSD enzymes is driven by TNF-α whereby inflammatory cytokines cause an autocrine switch in intracellular corticosteroid metabolism [36]. The plasticity of Th 17 cells in autoimmune inflammations was studied in much detail in many inflammatory diseases including type II reactions or erythema nodosum leprosum (ENL) [42]. IL-17A is consistently present in the skin tissues of cases with type II reactions before and during thalidomide treatment indicating an active role of IL-17 family of cytokines in skin immune surveillance. We observed an upsurge in IL-17F during type 1 reactions in leprosy [4]. IL-6 can be secreted by macrophages in response to specific M. leprae surface molecules, referred to as pathogen associated molecular patterns (PAMPs). These PAMPs bind to pattern recognition receptors (PRRs), including Toll-like receptors (TLRs). These are present on the cell surface and intracellular compartments and induce intracellular signalling cascades that give rise to inflammatory cytokine production. It has been demonstrated that IFN-γ and IL-6 levels increased during type 1 and type II reactional states in leprosy [43]. We observed a significant increase in IL-6 levels in cases with T1R when compared to NR cases.

We studied IL-7, IL-23 and IL-1 β to understand their circulatory levels in type 1 reactional states as these molecules are actively involved in acute inflammations. However we are not able to observe significant variations despite the earlier reports on IL-1 β [44] with 22 cases indicating an upsurge during type 1 reactions.

5. Conclusion

In conclusion, our study indicated an active association of urinary and lesional skin cortisol levels with the development of type 1 reactions in leprosy. Differential mRNA expression profiles of localized cortisol regulating enzymes (11 β HSD I & II) may throw some light on understanding the downstream functional implications of low cortisol availability in type 1 reactional skin lesions and the need for external administration of hydrocortisone. Proinflammatory cytokines – IL-17F, IP-10, IL-6 and TNF-α can act as potential serological as well as gene expression markers for early detection and diagnosis of type 1 reactions there by preventing nerve damage and consequent deformities in leprosy.

Conflict of interest

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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References

[16] Steensberg A, Fischer CP, Keller C, Moller K, Pedersen BK. IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. American Journal of Physiology,


