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Original Article

Nitric oxide metabolites in sera of patients across the spectrum of leprosy

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Leprosy, a chronic infectious disease, caused by *Mycobacterium leprae* infection, manifests itself as a clinical spectrum depending on the patients' immunological response, finally leading to peripheral nerve damage and deformities in the patients. Nitric oxide (NO) which is known to contribute to pathogenesis of several neurological diseases has been detected in tissues and urine of leprosy patients. This is the first study assessing NO as its stable end products, nitrites and nitrates, in sera of patients across the spectrum of the disease as a possible parameter of prognostic value. Comparison of NO metabolites showed a significant increase in multibacillary patients and patients with type I reactions as compared to healthy control individuals. These levels reduced significantly after treatment. This study has further borne out the utility and reliability of the cadmium-reduction method of estimation of NO metabolites - a relatively inexpensive procedure that lends itself to large-scale screening and follow-up of patients.

Key words : Leprosy, Nitric oxide, Serum, Cadmium-reduction method

Introduction

Leprosy is a chronic infectious disease caused by the obligate intracellular parasite *Mycobacterium leprae* that tends to infect skin and the peripheral nerves. Nerve damage seen across the spectrum of leprosy is the main cause of deformities and morbidity in this disease (Scollard et al 2006). The disease is seen in a variety of clinical forms as its manifestation is largely determined by the host immune response to the pathogen, ranging from a single, localized lesion (tuberculoid leprosy-TT) to widely disseminated infiltration by a number of organisms (lepromatous leprosy-LL). Between the two polar forms are the borderline types, forming a continuous immunological spectrum (Ridley and Jopling 1966). Borderline patients are immunologically unstable and may present with detrimental reactions called type 1 reactions (T1R) or reversal reactions (RR), often occurring concurrently with anti-leprosy treatment.

Nitric Oxide (NO) is a ubiquitous gaseous molecule that plays a role in several physiological functions including vasodilation, neurotransmission and inflammatory responses (Guix

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et al 2005). Production of reactive nitrogen intermediates, especially NO, by macrophages is an important mechanism for inhibition of several intracellular pathogens (MacMicking et al 1997). This is also observed in leprosy tissues (Visca et al 2002). However, under certain conditions, large amounts of NO are produced by the inducible isoform of the enzyme, nitric oxide synthase (iNOS) which contributes to pathogenesis of several neurodegenerative diseases such as multiple sclerosis, Parkinson's and Alzheimer's disease (Heales et al 1999, Acar et al 2003, Duncan and Heales 2005, Malinski 2007).

In leprosy, iNOS expression has been reported locally in skin biopsies across the spectrum of patients which is further increased in patients undergoing reversal reaction (Khanolkar-Young et al 1998, Schon et al 2001) but reduces on prednisolone treatment (Little et al 2001). Increase in NO metabolites have also been reported in the urine of leprosy patients undergoing reversal reactions (Schön et al 1999, 2000) and in serum and urine of patients with type 2 reaction (Rada et al 2003, Mohanty et al 2007) which decreased on successful resolution of the reactions (Mohanty et al 2007), however, this is the first report on NO metabolite levels in the serum of leprosy patients across the spectrum.

The present study has compared the NO levels, estimated as its stable end products (nitrates) NO_3 and (nitrites) NO_2 in the serum of leprosy patients across the spectrum with levels in healthy individuals, before and after treatment, using the cadmium reduction method.

Materials and Methods

Collection of blood samples

Blood samples were collected and sera separated from leprosy patients attending the clinic at the Foundation for Medical Research, Mumbai and stored at -20°C until use. A total of 86 sera samples across the leprosy spectrum were analyzed. The patients were diagnosed and classified according to the Ridley and Jopling classification (1966) based on clinical and histopathological findings. For this study, the patients were grouped into paucibacillary (PB) (n=33) if they had 5 or less skin lesions and multibacillary (MB) (n=25) if they had more than 5 skin lesions (WHO 2000). This functional classification was used since there were no patients with polar TT leprosy and only three patients who were polar LL. Within these groups patients were further separated into untreated or those treated with standard WHO recommended multidrug therapy (MDT) for not less than 6 months. Patients undergoing type 1 reaction (T1R) were treated as a separate group (n=28). Among this group patients who had undergone treatment with MDT and predisolone were considered as the treated group. Untreated patients had not been given any MDT treatment previously. The samples were coded and provided for estimation to ensure a blind study. Serum collected from healthy individuals of both sexes (n=24), age ranging from 21 to 50 years served as age matched controls. All protocols employed were approved by the Institutional Ethical Committee (IEC) and informed consent was taken from the patients.

Analysis of nitrate and nitrite

NO was estimated as NO_2 and NO_3 which are its stable end products of metabolism. Sera samples stored at –20°C were deproteinized using alcohol in a 1:2 ratio (Miranda et al 2001) centrifuged at 2000 rpm for 15 minutes and the supernatants were used for the assay. Nitrates were reduced to nitrites by the kinetic cadmium-reduction method (Cortas and Wakid 1990). Briefly, 0.5 ml of deproteinized sample was incubated with 0.5 ml glycine-NaOH buffer (200mM, pH 9.7) and approximately 1.5 gms of copper-coated cadmium granules for 90 minutes at room temperature. Total nitrites were then estimated by incubating 100 µl of the cadmium treated sample with 100 µl of Griess reagent [50 µl Sulphanilamide (2% in 5% HCl acid) + 50 ml Naphthylethylene diamine dihydrochroride (NEDD) (0.1%)] at room temperature for 30 minutes. Absorbance was then measured at

540 nm using an ELISA reader (Biotek). The amount of nitrite in the sera samples was obtained from a standard graph of $NaNO_2$ (0-25 μM) (Miranda et al 2001). As an internal control for the cadmium-reduction, with every set, standard amounts of KNO_3 were simultaneously reduced using cadmium to ascertain that the reduction was satisfactory.

Statistical analysis

Data was expressed as mean ± SD. Significance of differences between the groups was determined using a Mann Whitney U non-parametric test. Statistical significance was established at $p < 0.05$.

Results

NO metabolite levels in leprosy sera

Our study has estimated the NO metabolite levels in leprosy patients clinically classified into paucibacillary (5 or less skin lesions) and multibacillary (more than 5 lesions) cases both in untreated patients and after treatment with MDT for a minimum of 6 months (Figure 1). Since NO metabolite levels in sera and other body fluids vary among individuals, it was essential to first estimate the values in the healthy Indian population. Using the cadmium-reduction method the range in healthy individuals was found to be 40.57 ± 12.26 μM (n=24). A total of 86 leprosy sera across the spectrum both untreated and after MDT treatment were estimated for NO metabolites (Table 1 and Figure 1). Comparison of the means for each group of untreated patients with the value for the healthy population showed an increase across the spectrum, however, the increase was significant only for the MB group (67.65 ± 27.07 μM; $p<0.001$) and patients undergoing T1R (65.25 ± 29.55 μM; $p<0.001$). No correlation was observed with BI (Bacteriological Index) of the patient (data not shown). Following treatment with standard MDT, for a minimum of 6 months MB patients showed significantly reduced levels (40.82 ± 13.21 μM; $p<0.01$). Similarly, patients suffering from T1R also showed significant reduction in the levels (36.77 ± 18.88 μM; $p<0.01$) following treatment with MDT and prednisolone (Table 1). If T1R patients are separated into PB and MB groups, patients with MB leprosy showing clinical manifestation of T1R had a higher value of NO (77.53 ± 35.22) as compared to patients with PB leprosy (52.97 ±

Table 1 : Comparison of nitric oxide metabolite levels of leprosy patients across the clinical spectrum with healthy controls before and after treatment

Clinical status	Treatment status	No. of patients	Nitrite levels (μM) (Mean ± SD)
Normal (NN)		24	40.57 ± 12.26
Paucibacillary (TT/BT)	Untreated	18	47.52 ± 22.22
Paucibacillary (TT/BT)	Treated with MDT	15	35.45 ± 10.56
Multibacillary (LL/BL/BB)	Untreated	12	67.65 ± 27.07*
Multibacillary (LL/BL/BB)	Treated with MDT	13	40.82 ± 13.21#
Type 1 reaction	Untreated	18	65.25 ± 29.55*
Type 1 reaction	Treated with MDT and CoS	10	36.77 ± 18.88#

***p<0.001 as compared to control**

#p< 0.01 as compared with its untreated group

MDT : Multidrug therapy

CoS : Corticosteroid (prednisolone)

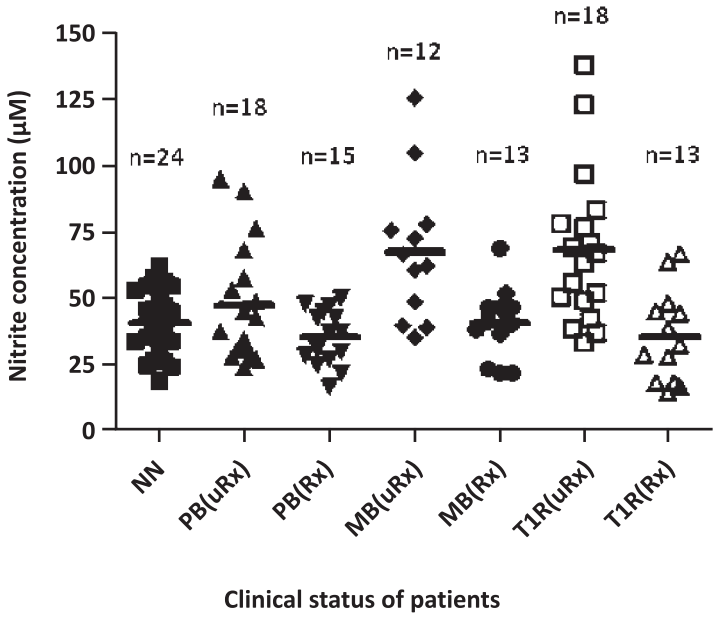

Figure 1 : Scattergram comparing the NO metabolites from leprosy patients across the spectrum with normal healthy individuals.

NN : Healthy normal individuals, P**B(uRx)** : Untreated paucibacillary patients, **PB(Rx)** : Treated paucibacillary patients, **MB(uRx)** : Untreated multibacillary patients, **MB(Rx)** : Treated multibacillary patients, **T1R (uRx)** : Untreated type 1 reaction, **T1R (Rx)** : Treated type 1 reaction

Table 2 : Estimation of NO metabolites (µM) in sera of 5 patients before and after recommended therapy

No	Clinical status	Untreated	Treated	Duration and type of treatment
1	MB (BL-LL)	105.0	38.4	6 m, MDT
2	MB (LL with ENL)	40.2 *	25.5	6 m, MDT + CoS
3	MB (BL with T1R)	50.4 *	18.3	12 m, MDT + 6 m CoS
4	PB (BT-BB with T1R)	52.2	38.4	1 m, MDT + CoS
5	PB (BT with T1R)	55.8	48.0	6 m, MDT + CoS

***These samples though considered untreated were taken after 15 days of prednisolone treatment**
MDT : Multidrug therapy
CoS : Corticosteroid (Prednisolone)

16.60). On treatment the levels of NO reduced in both types of patients.

Among the sera tested there were five patients from whom samples had been collected before and after treatment. The patients were from different clinical groups. Table 2 summarizes these follow up cases. These cases reiterate our observations that after treatment there is a reduction in the amount of NO metabolites in the serum.

Discussion

Leprosy is a chronic disease that manifests itself clinically as a spectrum depending on the individual's immunologic response to *M.leprae*. Several clinical and pathological parameters such as bacterial load, clinical manifestation and histopathological observations of lesions, onset and types of reactions and humoral and cellular immunity show opposing trends depending on which type of disease is manifested (Scollard et al 2006). Hence, any new parameter studied as an indicator of disease state needs to be determined in patients across the spectrum.

NO, a small diffusible gaseous molecule is implicated in the pathogenesis of several diseases including multiple sclerosis, Parkinson's and Alzheimer's disease (Heales et al 1999, Acar et al 2003, Duncan and Heales 2005, Malinski 2007). In all these diseases, increased levels on NO metabolites have been detected in body fluids such as serum/plasma, urine or CSF (Rejdak et al 2008). Generation of reactive nitrogen intermediates, especially NO, by macrophages is an effective anti-microbial mechanism against intracellular pathogens including *M. tuberculosis* (Adams et al 1997) and *M.leprae* (Visca et al 2002). Activation of macrophages by pro-inflammatory cytokines like IFN-γ is known to induce the production of NO through iNOS (Cooper et al 2002). This explains the high levels of NO and iNOS in patients with T1R where levels of IFN-γ and IL-12 are also increased (Little et al 2001). Our study further corroborates this observation with maximum levels of NO metabolites obtained in sera of patients with T1R. Treatment with steroids is known to reduce cytokine production mainly through inhibition of NF-κB – induced transcription of the cytokines (Scheinman et al 1995) which would lead to a reduction in NO levels. Inhibition of NF-κB is also known to directly inhibit iNOS activity (Guix et al 2005). Both these pathways will lead to reduction in NO levels in sera in patients treated with prednisolone.

In leprosy, iNOS, the enzyme responsible for NO production, has been localized in skin lesions especially in patients with TT and T1R (Khanolkar-Young et al 1998). NO metabolites have also been estimated in biological samples, mainly in patients undergoing reactions. Schön et al (1999) have reported an increase in NO metabolites in urine of patients with T1R and further showed a rapid decrease in patients responding to predinsolone treatment (Schön et al 2000). Similarly an increase in NO metabolites has been shown in sera (Rada et al 2003) and urine (Mohanty et al 2007) of patients with type 2 reactions but no reports on the levels of NO metabolites in sera of patients across the spectrum is available.

Our study has estimated the NO metabolite levels in leprosy patients clinically classified into PB and MB (based on number of skin lesions) cases both in untreated patients and after treatment with multidrug therapy for a minimum of 6 months. Our results show highest levels in the untreated multibacillary patients and in patients undergoing T1R. This is in contrast to the maximum localization of the iNOS enzyme in skin lesions of TT patients (Khanolkar-Young et al 1998). Since iNOS is induced during inflammatory responses its increased expression in localized TT skin lesions is expected, however, the serum values reflect the metabolic status of the entire body and hence NO metabolite levels are presumably higher in multibacillary patients with chronic multiple lesions. Follow-up of five patients

confirmed reduction of NO levels after treatment. Our study also showed that treatment reduced the levels of NO metabolites, not only for T1R patients, as has been reported earlier (Schön et al 2000, Little et al 2001) but also for patients without reactions.

In conclusion, this study indicates that NO levels in sera of leprosy patients increase during the disease and partially correlate with the clinical status of the patient with values being higher in MB patients and patients with T1R. Since our study shows that clinical improvement with successful treatment decreases the NO levels significantly, this method could be evaluated further to monitor treatment success especially in MB and patients with reactions. Further, in this study; we have standardized the estimation of NO metabolites using Cu-coated cadmium for reduction of NO_3 to NO_2, thus, eliminating the need for expensive enzymes.

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