

## Increased levels of nitric oxide metabolites in urine from leprosy patients in reversal reaction

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**Summary** We measured the metabolites of NO [nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ )] in urine from Ethiopian patients suffering from leprosy. The urinary level of  $\text{NO}_2^-/\text{NO}_3^-$  in a group of healthy Ethiopians was  $1020 \pm 471 \mu\text{M}$  ( $n = 22$ ). Leprosy patients in reversal reaction had significantly higher levels of  $\text{NO}_2^-/\text{NO}_3^-$  ( $1817 \pm 492 \mu\text{M}$ ,  $p < 0.001$ ,  $n = 12$ ) than both the control group and leprosy patients who were not in reversal reaction ( $1079 \pm 446 \mu\text{M}$ ,  $n = 12$ ). We conclude that the reversal reaction in leprosy is associated with increased urinary levels of nitric oxide metabolites.

### Introduction

Leprosy according to WHO definitions presently affects approximately 1 million individuals worldwide<sup>1</sup> and the disease primary involves the skin and peripheral nerve system.<sup>2</sup> Leprosy is classified according to immunity as a polar disease with a spectrum ranging from the tuberculoid (TT) to the lepromatous (LL) form, with the borderline forms in between.<sup>3</sup> The term 'reaction' in leprosy is used to describe the appearance of symptoms and signs of acute inflammation. Type 1 reversal reactions occurring in borderline cases are associated with an increase in cell-mediated immunity, and nerve damage from neuritis may be rapid and severe.<sup>2</sup>

Inducible NO synthase (iNOS) is responsible for the synthesis of NO and L-citrulline from L-arginine by activated macrophages. NO is highly unstable and decays to its stable end products nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ), which are eliminated in the urine.<sup>4</sup> Evidence is accumulating that nitric oxide (NO) produced by macrophages is an important factor in host defence against intracellular pathogens such as *M. tuberculosis*<sup>5–7</sup> and *M. leprae*.<sup>8,9</sup> A recent

report has described the presence of iNOS in skin biopsies from patients undergoing reversal reaction.<sup>10</sup> The aim of this study was to investigate the urinary levels of nitric oxide metabolites in patients with leprosy in the reactional stage of the disease.

## Materials and methods

### MATERIALS

These were: *Aspergillus* nitrate reductase (Boehringer Mannheim, Freiberg, Germany), NADPH as the tetrasodium salt (Boehringer Mannheim), sulphanic acid (Merck, Darmstadt, Germany), sodium nitrate (Merck), sodium nitrate (Sigma Chemical Co., St Louis, USA), *N*-(1-naphthyl) ethylenediamine (Sigma) and phosphate buffered saline (PBS).

### COLLECTION OF URINE SAMPLES

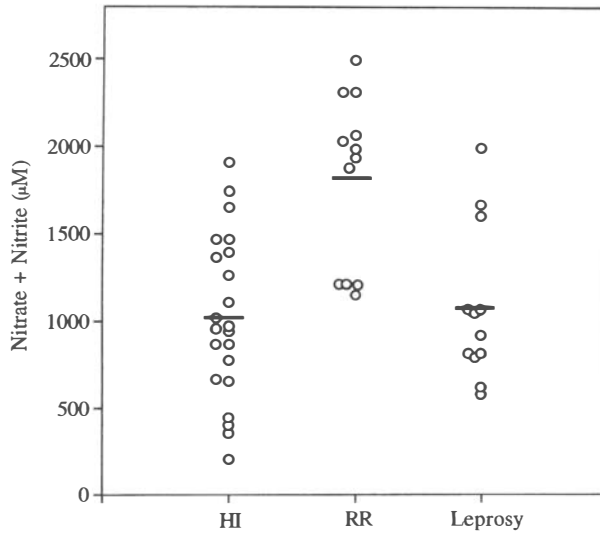
Leprosy patients in the reactional stage were admitted to the wards of ALERT Hospital, Addis Ababa, Ethiopia because of reversal reaction in leprosy [ $n = 12$ , males = 7, females = 5, average age  $31 \pm 12$  years old, age range 14–54, borderline lepromatous (BL = 8), borderline tuberculoid (BT = 4)]. All patients were in reversal reaction (type I reaction) and were differentiated clinically from patients with erythema nodosum leprosum (type II reaction). The samples from these patients were obtained within 2 weeks after admission and all patients were on steroid treatment. The patients did not show clinical signs of any co-existing disease and all had negative stool samples by microscopy. Urine samples were also collected from 12 patients suffering from borderline forms of leprosy [borderline lepromatous (BL = 2), borderline tuberculoid (BT = 10)] who were not undergoing reversal reaction (males = 5, females = 7, average age  $38 \pm 14$  years old, age range 18–63). The control group for urinary levels of  $\text{NO}_2^-/\text{NO}_3^-$  consisted of 22 healthy Ethiopian individuals ( $n = 22$ , males = 13, females = 9, average age  $36 \pm 9$  years old, age range 20–55). All urine samples were morning urine samples and were stored at  $-20^\circ\text{C}$  until analysed. The study was approved by the ethical committee at Armauer Hansen Research Institute and ALERT Hospital, Addis Ababa, Ethiopia.

### ANALYSIS OF NITRITE AND NITRATE

The sum of the  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations in urine was determined essentially as described by Giliam *et al.*<sup>11</sup> Urine was diluted in PBS. Nitrate in the samples was reduced to  $\text{NO}_2^-$  by incubating the sample with  $10 \mu\text{l}$  nitrate reductase from *Aspergillus* (10 IU/ml) and  $10 \mu\text{l}$  nicotinamide adenine dinucleotide phosphate (reduced NADPH; 1 mM) for 2 h at  $37^\circ\text{C}$ . The  $\text{NO}_2^-$  level was then determined by the Griess reaction. The final reaction volume of 1 ml contained  $728 \mu\text{l}$  HCl (0.45 M),  $86 \mu\text{l}$  sulphanic acid (2 mg/ml),  $86 \mu\text{l}$  *N*-(1-naphthyl) ethylenediamine (1 mg/ml) and  $100 \mu\text{l}$  of the diluted urine sample. The urine samples were then analysed on a ELISA multiwell reader (Titertec Multiscan Plus) at 542 nm.

### STATISTICAL ANALYSIS

Statistical evaluation was performed with the Mann–Whitney *U*-test for comparison between groups. Data are presented as mean  $\pm$  SD.



**Figure 1.** Urinary levels of nitrate and nitrite (the mean is represented by bold lines) in: healthy Ethiopian individuals (HI,  $n = 22$ ), leprosy patients in reversal reaction (RR,  $n = 12$ ) and leprosy patients who were not undergoing reversal reaction (Leprosy,  $n = 12$ ).

## Results

The control group consisted of apparently healthy Ethiopian individuals. The mean urinary excretion of  $\text{NO}_2^-/\text{NO}_3^-$  in this group of 22 individuals was found to be  $1020 \pm 471 \mu\text{M}$  (range 198–1907) (Figure 1). Urine was obtained from leprosy patients admitted to the ALERT Hospital, Addis Ababa, Ethiopia because they were undergoing reversal reaction. Leprosy patients in reversal reaction had significantly higher levels of  $\text{NO}_2^-/\text{NO}_3^-$  ( $1817 \pm 492 \mu\text{M}$ ,  $p < 0.001$ ,  $n = 12$ ) than both the control group and leprosy patients who were not undergoing reversal reaction ( $1079 \pm 446 \mu\text{M}$ ,  $n = 12$ ).

## Discussion

Nitric oxide produced by the macrophage has been suggested to play an important role in the host response to pathogens targeting the macrophage such as *M. leprae*.<sup>10,12,13</sup> The metabolites of NO,  $\text{NO}_3^-$  and  $\text{NO}_2^-$ , have been used as a measurement of the production of NO in serum and urine from humans in various diseases such as cancer,<sup>14</sup> malaria<sup>15</sup> and children with coeliac disease.<sup>16</sup> In humans the sources of  $\text{NO}_2^-/\text{NO}_3^-$  are food, air ( $\text{NO}_2$ ) and endogenous synthesis.<sup>17</sup> In this study, there were no restrictions of  $\text{NO}_2^-/\text{NO}_3^-$  in the diet in any of the study groups, and increases of  $\text{NO}_2^-/\text{NO}_3^-$  might be influenced by a diet rich in nitrate-containing food. However, in this study there was a strong correlation between increased levels of NO metabolites and the reactional stage of leprosy, where NO has been suggested to play a role in the host response. Our results are also consistent with a recent report where 13 out of 15 leprosy patients undergoing reversal reaction were iNOS positive in skin biopsies, whereas BT patients and healthy controls were completely negative.<sup>10</sup> The

reactional stage of leprosy is a stage of the disease associated with acute inflammation.<sup>2</sup> Patients with ulcerative colitis, Crohn's disease and coeliac disease also have increased levels of nitrate and nitrite during active stages when the degree of inflammation is high, but normal levels of nitrate and nitrite between these stages.<sup>16,18</sup> Thus, there seem to be a connection between NO production and acute inflammation. Further studies are required to evaluate raised levels of urinary NO metabolites as a predictive marker of reversal reactions in leprosy. We conclude that leprosy patients in reversal reaction have increased urinary levels of NO metabolites.

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