

Release of reactive nitrogen intermediates from the peripheral blood-derived monocytes/macrophages of leprosy patients stimulated *in vitro* by tuftsin

S. KHARE, L. K. BHUTANI & D. N. RAO*

*Department of Biochemistry and Department of Dermatovenereology
All India Institute of Medical Sciences, New Delhi-110 029, India*

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Summary The production of reactive nitrogen intermediates (RNI) by macrophages is critical to host defence, particularly for exerting the bactericidal and tumoricidal properties. Nitric oxide (NO) were measured in the peripheral blood-derived monocytes/macrophages of normal and leprosy patients (BT/TT and BL/LL) in the presence and absence of 'tuftsin' as a function of *in vitro* culture age (on 1, 3, 7 days). Macrophages from both groups of leprosy patients were able to produce NO during the unstimulated state but only BL/LL macrophages could be activated by tuftsin to produce significantly high levels of NO. This increase was highest on day 1, then gradually decreased with *in vitro* culture age. Surprisingly, tuftsin was unable to enhance the NO production in normal macrophages above the basal level. Further, normal and BT/TT macrophages had only Cu–Zn derived superoxide dismutase (SOD) activity whereas BL/LL cultures has Cu–Zn and Mn derived SOD activity. These studies indicate that in BL/LL cultures: a, apart from tuftsin, some additional signal is required to activate nitric oxide synthase (NOS) gene for NO production; and b, Mn–SOD produced by *Mycobacterium leprae* is playing a defensive role against toxic-free radicals. The final outcome of this mechanism is the survival of *M. leprae* inside the macrophages.

Introduction

Activated macrophages play an important role in host resistance to the development of clinical leprosy and in the limitation of growth of *Mycobacterium leprae*. Tuftsin, a tetrapeptide from immunoglobulin G, is an endogenous molecule and a stimulator of phagocytic and microbicidal properties of macrophages has enormous potential clinical implications.^{1–3} This immunomodulator exerts a differential effect for the phagocytic and microbicidal activity in the monocytes/macrophages derived from peripheral blood of leprosy patients.^{4,5} It was able to stimulate macrophages of the tuberculoid group for the microbicidal activity but failed to show similar effect(s) in the lepromatous leprosy group. Furthermore, it

* Corresponding author

was ineffective in modulating the superoxide anion (O_2^-) production, but was effective in enhancing the production of hydrogen peroxide (H_2O_2) from the monocyte/macrophages of lepromatous leprosy patients.⁶ Studies concerning the mechanism of intracellular killing of pathogens such as leishmania, toxoplasma, *M. leprae*, *M. tuberculosis* and trypanosomes using the murine system *in vivo* and *in vitro* have suggested that production of reactive nitrogen intermediates (RNI) may be the chief pathway of killing by macrophages stimulated with interferon-gamma (IFN- γ) or related cytokines.⁷ The activated state of macrophages (as defined by their ability to induce intracellular killing) is related to the release of reactive oxygen as well as nitrogen intermediates and the interaction between them is essential for the final outcome of microbicidal process in the form of lethal radical production. Likewise, the ability of the intracellular pathogens to survive inside the macrophages is related to their levels of scavenging enzymes, amongst them superoxide dismutase (SOD) is one of the key enzymes. Superoxide dismutase is also known to enhance the apparent generation of nitric oxide (NO) from L-Arginine without directly affecting the chemical stability of NO itself.⁸ The importance of RNI as a causative agent in the killing mechanism is still not clear in the human blood-derived macrophages. The survey of literature indicates that the production of inducible nitric oxide synthase (NOS) is highly regulated and a delicate functional balance among various microbial stimuli, host-derived cytokines and other factors in the micro-environment is thought to be very important for this regulation.

Modulating the production of reactive intermediates (superoxide anion, hydrogen peroxide, hydroxyl radicals and nitric oxide) and their interaction may contribute to the ability of *M. leprae* either to survive or to be susceptible to these toxic radicals. Hence, the present study is designed to investigate the effect of tuftsin for the RNI production in monocyte/macrophages derived from peripheral blood of normal individuals and leprosy patients as a function of *in vitro* culture age. Furthermore, the present study delineates the mechanism(s) underlying the microbicidal process across the spectrum of leprosy during activation of monocytes/macrophages.

Materials and methods

COLLECTION OF BLOOD SAMPLES

Leprosy patients were classified in two groups, i.e. tuberculoid (BT/TT) and lepromatous leprosy (BL/LL) according to their clinical, bacteriological and histological findings.⁹ None of the patients had received any previous anti-leprosy treatment. Blood samples were collected in heparinized sterile tubes from these patients and normal healthy individuals, who were taken as a control group and had no previous contact with leprosy patients. A total of 7 normal, 7 BT/TT and 6 BL/LL individuals were assayed for both RNI production and SOD activity.

ISOLATION AND CULTURING OF MONONUCLEAR CELLS

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation on histopaque (density 1.077 g/ml).⁶ On an average $1-2 \times 10^6$ PBMC/ml of whole blood, containing 10-15% adherent macrophages were obtained by the above procedure. The viability (by Trypan blue exclusion dye) and purity of cells (by nonspecific esterase staining) were found to be more than 95%.

NITRITE PRODUCTION

On the appropriate day of the culture (1, 3 and 7 day), 1×10^5 cells/well were plated in a 96-well tissue-culture plate (Linbro, Flow Laboratories) and were stimulated with the optimal concentration of tuftsin ($0.88 \mu\text{M}$) for an optimum time period (24 h) at 37°C . Another set of culture was kept unstimulated. During the assay period (24 h), cells were kept in HBSS medium containing arginine (2 mM) and glutamine (2 mM). After the incubation, supernatants were transferred to another microtitre plate and kept frozen at -20°C for testing later for nitrite production by Griess reagent.¹⁰ The results were expressed as μM nitrite/ 10^5 cells/24 h.

SUPEROXIDE DISMUTASE ACTIVITY

Total SOD, i.e. human macrophages derived (copper-zinc linked) plus mycobacterial derived (manganese linked) and alone Mn-linked SOD activity were measured. Mn-linked SOD activity was measured by inhibiting the Cu-Zn-linked SOD activity using 1 mM potassium cyanide (KCN). On the appropriate day of assay, 5×10^5 cells/well were transferred to a 96-well flat-bottom plate. One set of cells were incubated for 30 min with 1 mM KCN and other set of cells were incubated in HBSS media alone. The cells were lysed by adding $10 \mu\text{l}$ of 0.2% triton X-100. The cell lysates were centrifuged at 4°C for 20 min at $1500 \times g$ to remove cellular debris. Supernatants were transferred into another plate and was stored at -20°C till further use. Superoxide dismutase (SOD) activity was measured spectrophotometrically by its ability to inhibit the superoxide anion mediated reduction of ferric cytochrome c by the Xanthine-Xanthine Oxidase system.¹¹

STATISTICAL ANALYSIS

All the samples were run in triplicate. Results were expressed as mean \pm SD of samples. To determine the difference within a group (in unstimulated and stimulated) 'Student's *t* test' was used. To evaluate the intergroup differences in individuals (Normal, BT/TT and BL/LL) and the day of culture (1, 3 and 7 day) one way analysis of variance (ANOVA) was used. 'Multiple range test' was used to calculate pairwise significance between two subgroups.

Results

A history of each patient for their clinical, histopathological and bacterial index are given in Table 1.

While standardizing the optimal experimental conditions for NO production a requirement of exogenous L-arginine (2 mM) in the HBSS medium was found to be essential. Glutamine (2 mM) was also added in the culture medium to maintain the cell viability for 24 h. Tuftsin at a concentration of $0.88 \mu\text{M}$ and an incubation period of 24 h was found to be optimum for nitrite production.

NITRITE PRODUCTION IN NORMAL HEALTHY INDIVIDUALS AND LEPROSY PATIENTS

The basal levels (unstimulated) of nitrite production in 1-day-old cultures of both the groups of the leprosy patients were found to be significantly ($p < 0.001$) higher than the normal

Table 1. Detailed history of each patient for clinical, histopathological and bacterial index

Group	S. No.	Patient Code No.	Clinical diagnosis	Histological diagnosis	Bacterial index	Mn-SOD activity (U/ $\times 10^5$ cells)		
						1 day	3 day	7 day
BL/LL	23	L23	BL	BL	4+	0.130	0.268	0.672
	24	L24	BL	-	4+	0.128	0.257	0.661
	25	L25	LL	LL	5+	0.164	0.319	0.770
	26	L26	LL	BL	5+	0.142	0.264	0.660
	27	L27	BL	BL	4+	0.143	0.276	0.677
	28	L28	LL	LL	6+	0.170	0.332	0.720
BT/TT	29	T29	BT	BT	1+	0.021	0.019	0.009
	30	T30	BT	TT	0	0.022	0.031	0.021
	31	T31	BT	BT	0	0.031	0.028	0.019
	32	T32	BT	BT	0	0.019	0.014	0.015
	33	T33	BT	BT	1+	0.017	0.011	0.011
	34	T34	BT	BT	1+	0.000	0.000	0.000
	35	T35	BT	BT	2+	0.007	0.012	0.011

individuals (Figure 1). Upon stimulation with $0.88 \mu\text{M}$ tuftsin, normal and BT/TT monocytes were unable to undergo any stimulation for nitrite production; whereas BL/LL monocytes showed a significant ($p < 0.001$) increase in nitrite production. On day 3 of culture, the normal macrophages had again significantly ($p < 0.05$) low levels of nitrite production as

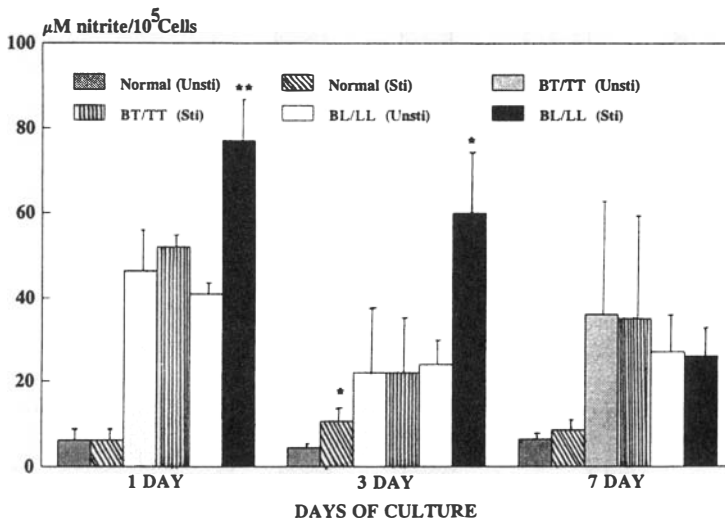


Figure 1. Nitrite levels in normal individuals and leprosy patients: Levels of nitrite during unstimulated (Unsti) and stimulated (Sti) state in the peripheral blood-derived monocytes/macrophages of normal healthy individuals and leprosy patients, during *in vitro* culture age. Stimulant (tuftsin = $0.88 \mu\text{M}$) was incubated with monocytes/macrophages for 24 h in the presence of HBSS + Arg (2 mM) and Gln (2 mM) and then culture supernatant (in triplicate for each sample) were collected. Results are mean \pm SD of number of samples (normal, BT/TT $n = 7$ and BL/LL $n = 6$). Values of significance (*, $p < 0.01$; and **, $p < 0.001$) are of comparison between unstimulated and stimulated state.

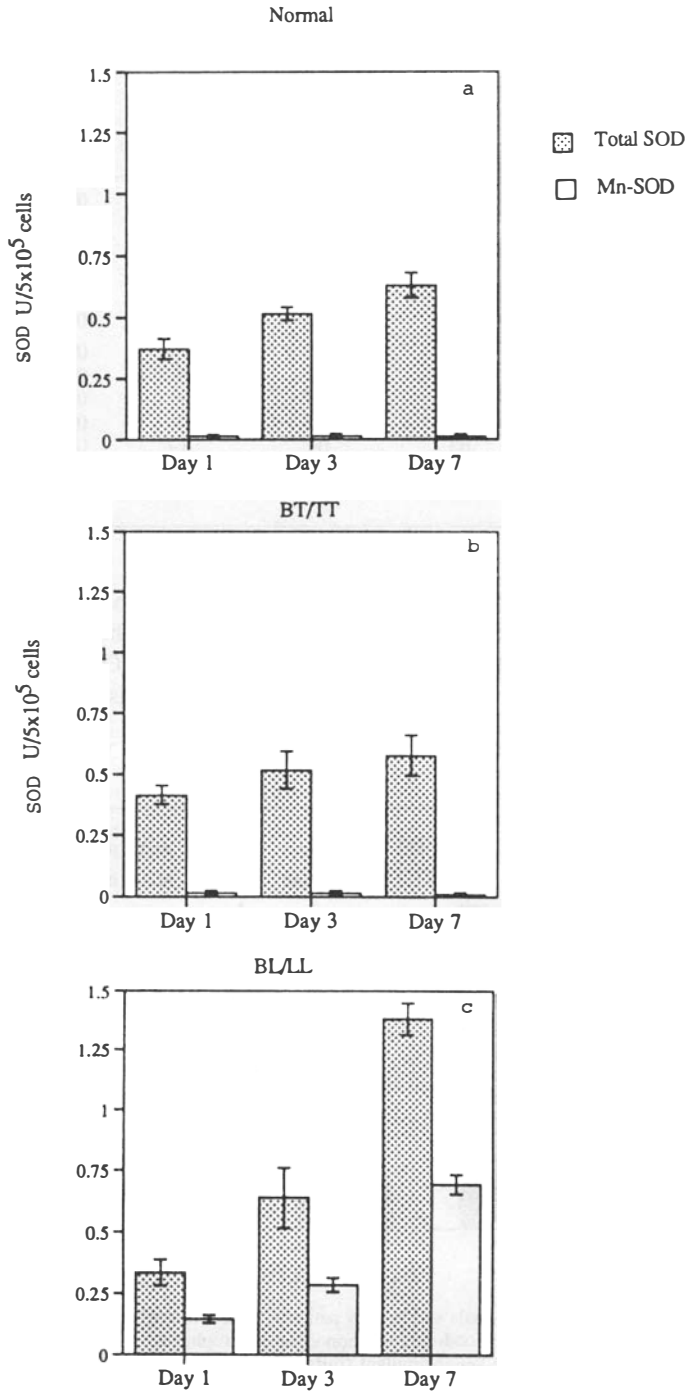


Figure 2. Activity of superoxide dismutase in monocytes/macrophages of normal healthy individuals (2a) and leprosy patients (2b, 2c) during *in vitro* culture age. Mn-linked SOD was determined by inhibiting Cu–Zn SOD with 1 mM KCN. Each sample was run in triplicate and results expressed as mean \pm SD of number of samples (normal, BT/TT $n = 7$ and BL/LL $n = 6$).

compared to both the groups of leprosy patients. On day 3 upon stimulation with tuftsin, normal and BL/LL groups showed a significant ($p < 0.01$) increase in the nitrite production, whereas BT/TT macrophages were unable to show any stimulation for nitrite production. The basal levels of nitrite production on day 7 in normal macrophages were also lower albeit insignificant as compared to BT/TT or BL/LL. Upon stimulation, macrophages from none of the groups showed any increase in the nitrite production on day 7 of culture. In general, while comparing the nitrite production with the *in vitro* culture age, normal and BT/TT monocytes/macrophages do not reveal any statistical changes. However, in the lepromatous leprosy group a gradual significant ($p < 0.05$) decrease was observed.

SUPEROXIDE DISMUTASE ACTIVITY

The total SOD enzyme activity (Cu-Zn-linked and Mn-linked) on day 1 was almost comparable in all the three groups (Figure 2). The Mn-linked SOD enzyme activity was detectable in the BL/LL cultures only and it contributed about 44% of the total SOD enzyme activity. Although on day 3, the total SOD enzyme activity was found to be almost comparable in all the three groups but the activity was significantly higher as compared to day 1. Again in BL/LL macrophages about 44.5% of the SOD enzyme activity was Mn-linked. On day 7, the normal and BT/TT macrophage cultures had almost the same total SOD enzyme activity, but the BL/LL cultures had significantly ($p < 0.01$) higher total SOD enzyme activity in which Mn-linked SOD activity was about 50%. A significant increase in the total SOD enzyme activity was observed in the normal ($p < 0.001$), BT/TT ($p < 0.05$) and BL/LL ($p < 0.001$) macrophages as the cell matured. Mn-linked SOD activity was observed in BL/LL monocytes/macrophages only, increased significantly ($p < 0.001$) with the age of the culture.

Discussion

Under normal circumstances human macrophages do not possess detectable NOS but stimuli such as IFN- γ and lipopolysaccharide (LPS) elicit nitric oxide synthesis over a few hours.¹² Since nitrite was measured after 24-h incubation and the existence of L-arginine within the macrophage is short lived an essential requirement for arginine in the HBSS medium was needed in the present study.¹³

At any day of the culture, the normal monocytes/macrophages showed the lowest levels of basal nitrite release as compared to both the groups of leprosy. No significant increase was seen in normal monocytes/macrophages even after stimulation with tuftsin. Thus it appears that apart from tuftsin some additional signal/factor is needed to activate nitric oxide synthase (NOS) gene for nitrite production. Macrophages infected with viable intracellular pathogen alone or in combination with IFN- γ or whole killed bacterial particles in the presence of interferon beta (IFN- β) has been shown to produce nitrite.^{14,15} Recently it has been reported that NO can also be produced during the infection of macrophages with Gram-positive bacteria.¹⁶ Macrophages activated with bacterial stimuli, can secrete a variety of cytokines, including tumor necrosis factor-alpha (TNF- α) and IFN- β .^{17,18} These cytokines function as autocrine or paracrine regulators of macrophage activation in terms of lethal toxic radicals generation.

In the present study both the groups of leprosy patients were able to produce nitrite during

the unstimulated state but only BL/LL monocytes/macrophages could be activated by tuftsin to produce significantly high levels of nitrite. In this connection the most interesting finding in the present study was the high activity of Mn-SOD which was found only in BL/LL monocytes/macrophages. Most of the Mn-SOD is known to be contributed by *M. leprae*.^{19,20} We have made an attempt to correlate the bacterial index (BI) and the Mn-SOD activity of the same patient by calculating the correlation coefficient (r). As Mn-SOD activity was detectable in BL/LL patients, r was restricted to these patients only. Mn-SOD activity in 1- and 3-day-old macrophages showed a strong positive correlation with bacterial index ($r = 0.866, 0.802$ respectively) which was statistically significant ($p < 0.05$). Mn-SOD activity in 7-day-old macrophages showed a weak positive correlation with BI ($r = 0.544$) and is statistically insignificant. The increase in Mn-SOD in the lepromatous leprosy group with age of the culture of the monocytes/macrophages could be due to the multiplication/growth of *M. leprae* inside the monocytes, a similar observation consistent with other workers.^{21,22} The paradoxical relationship between the multiplication period of *M. lepra* (11–13 days) and the increase in SOD activity during the *in vitro* culture age (1, 3 and 7 days) of monocyte/macrophages can be explained by the fact that *M. leprae* that are phagocytosed by circulating monocytes/macrophages are not in a synchronous state of multiplication. The present study suggests that a triggering signal provided by bacteria/bacterial particles is necessary for RNI production by the macrophages and we presume that in leprosy patients the monocytes are already primed *in vivo* by *M. leprae*. Tuftsin has been shown to induce NOS gene expression and produce NO in murine macrophages and has been found to replace the effects of LPS in IFN- γ induced NOS expression.²³ It has been observed that upon activation with tuftsin, macrophages generally required longer incubation time to synthesize new proteins.²⁴ It is possible that the effect of tuftsin is to increase cytokine(s) (TNF- α and IL-1 β) secretion which in turn, acts synergistically with the signal provided by bacteria or bacterial products to induce NOS gene.

The highest levels of nitrite was observed in BL/LL monocytes/macrophages on day 1 and then tapered off with the age of culture, this may be due to the inhibition of NO synthase activity by NO itself. This feedback inhibition could account for the rapid decline of the NO synthase activity once high levels of NO are generated. This self-regulatory pathway may be an effective mechanism for avoiding excessive production of NO, which can result in a range of pathological effects including macrophage cytotoxicity. Macrophages which are not initially overstimulated can indeed be repeatedly reactivated to induce the expression of NO synthase gene.²⁵ Hence, it is likely that this self-regulatory mechanism will enable the macrophages to exert their cytotoxic effect on target cells without damaging themselves in the process and will allow their reactivation, if required.

Despite the fact that BL/LL monocytes/macrophages are able to produce high nitrite levels either during the stimulated or unstimulated state, the earlier findings of our laboratory reporting the failure of tuftsin to inhibit the multiplication of *M. leprae* by BL/LL monocytes/macrophages cannot be neglected.⁵ An attempt has been made in the present study to investigate the mechanism of tuftsin action to correlate the interaction amongst the reactive free radicals and the microbicidal responses.

Normal and leprosy macrophages produce similar basal levels of O_2^- production but only leprosy macrophages produced NO_2^- . Superoxide dismutase activity (Mn-derived) was found only in BL/LL cultures. Superoxide anion is known to react with NO to form peroxynitrite anion, which decomposes rapidly on protonation to form OH^\cdot and NO_2^- and subsequently nitrate and SOD have been shown to increase the stability of this RNI by scavenging O_2^- .²⁶

Therefore, in BL/LL patients it is possible that SOD, by virtue of its ability to scavenge O_2^- , stabilizes the basal level of NO release. However, upon nonavailability of O_2^- , nitric oxide is nontoxic by itself; whereas in BT/TT both the toxic ions are present and upon interaction they produce more toxic ions that are lethal to *M. leprae*. The Mn-SOD could function in the defence against macrophage in two ways:

at an early time before the activation of monocytes/macrophages with tuftsin, substantial amounts of nitric oxide is generated, but the Mn-SOD is likely to scavenge superoxide anion production; and

during stimulation of monocyte/macrophage with tuftsin, the nitric oxide flux increases, and the destruction of O_2^- by the enzyme Mn-SOD may block the formation of intracellular peroxy nitrite anion by eliminating one of the reactants.

Thus, the final outcome is survival of *M. leprae* in BL/LL monocytes/macrophages by mitigating the effects of the mixture generated by macrophages rather than the toxicity of any individual component.

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