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Research Article

Indices of Phagocytosis in the Sputum and Peripheral Blood Mononuclear Cells Lysate of Pulmonary Tuberculosis Patients before Commencement of Chemotherapy

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Abstract

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by phagocytes are important in the control of infections including *Mycobacterium tuberculosis* (Mtb). Complete understanding of the involvement of these reactive species may be applied as therapeutic targets for tuberculosis control and management. This study assessed components of respiratory burst and nitric oxide in sputum and monocyte lysate of Nigerian tuberculosis (TB) patients before commencement of anti-TB chemotherapy.

160 participants were recruited for this study, comprising 50 multi-drug resistant TB (MDR-TB) patients, 60 drug-sensitive TB (DS-TB) patients and 50 non-TB apparently healthy individuals. Peripheral blood mononuclear cells (PBMCs) were obtained from blood using Ficoll density gradient method. Lysate was obtained from PBMCs by freeze thaw method. Spot sputum sample was collected in sterile universal bottle. Superoxide dismutase, catalase and myeloperoxidase activities as well as hydrogen peroxide and nitric oxide levels were assessed in sputum and lysate from PBMCs.

Sputum SOD activity was significantly increased in MDR-TB patients, though significantly decreased in DS-TB patients compared with controls. There were significant increases in sputum H_2O_2 level and sputum MPO activity in both MDR-TB and DS-TB patients compared with controls. Sputum NO level was also significantly increased in MDR-TB patients compared with controls. MDR-TB patients had significantly increased sputum SOD activity, H_2O_2 and NO levels compared with DS-TB patients. There were significant decreases in SOD activity and NO levels in lysate of DS-TB and MDR-TB patients respectively when compared to controls. Though SOD activity was significantly raised, there were significant decreases in lysate H_2O_2, NO and MPO activity in MDR-TB patients when compared with DS-TB patients.

The significantly raised levels of ROS and RNS in the sputum of TB patients might represents innate immune mechanisms to prevent spread or progression of Mtb infection in the local environment of the host, however it also indicates effective ROS/RNS evading mechanisms of Mtb.

Keywords: *Phagocytosis; Tuberculosis; Sputum; Peripheral Blood Mononuclear Cells; Progression*

Abbreviations

ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen Species; Mtb: *Mycobacterium tuberculosis*; TB: Tuberculosis; MDR-TB: Multi-Drug Resistant Tuberculosis; DS-TB: Drug Sensitive Tuberculosis; PBMC: Peripheral Blood Mononuclear Cell; SOD: Superoxide Dismutase; CAT: Catalase; MPO: Myeloperoxidase; NO: Nitric Oxide; HIV/AIDS: Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome; PMN: Polymorphonuclear Neutrophils; PAMP: Pathogen Associated Molecular Pattern; iNOS: Inducible Nitric Oxide Synthase; UCH: University College Hospital; PBS: Phosphate Buffered Saline; NOX2: NADPH Oxidase 2

Introduction

The clinical manifestation of tuberculosis (TB) caused by *Mycobacterium tuberculosis* (Mtb) depends on a combination of factors including host-pathogen interactions. The nature and course of these interactions is largely determined by the host immune system as demonstrated by the TB and HIV/AIDS synergy [1]. Hence, a clear understanding of the immune response to Mtb infection is crucial for identifying mechanisms that confer protection and shortfalls that lead to disease. This understanding will facilitate the development of novel vaccination strategies and identification of targets for adjuvant immunotherapeutic intervention.

Phagocytosis is a hallmark of anti-bacterial host defense and plays an important role in protection against Mtb. Mtb that successfully evades the physical barriers of the upper airways will be delivered to the alveoli where type II epithelial cells, alveolar macrophages and dendritic cells ingest the bacilli in a bid to contain infection [2]. This sets up an inflammatory response thereby recruiting other immune cells to the lungs, the highly motile polymorphonuclear neutrophils (PMNs) being the first responders and the predominant cell type infected in the airways of individuals with active TB [3,4]. Together, these professional phagocytes utilize the antimicrobial mechanisms at their disposal to kill ingested bacilli and bacilli within alveolar spaces.

Macrophages play a central role in mycobacterial pathogenesis; through recognition of Mtb pathogen associated molecular patterns (PAMPs), initiation of early induced cellular innate immune response, recruitment of other immune cells and stimulation of adaptive immune response, while also serving as a major cellular niche for bacterial replication during early infection and a reservoir for persistent bacteria within the lung granulomas during chronic infection [5-7]. Multiple mechanisms used by macrophages to eliminate Mtb have been reported, including the production of oxygen and nitrogen components, phagosome acidification and the autophagy of intracellular Mtb, among other processes [6]. Nonetheless, the specific role of these various mechanisms and their interactions with respect to Mtb infection progression are not fully understood.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by host phagocytic cells during innate immune response to infection are critical for early clearance and control of Mtb [8-10]. ROS produced in respiratory burst pathway includes superoxide radical, hydroxyl radical, hydrogen peroxide and hypochlorous acid. RNS are produced via the machinery of the inducible nitric oxide synthase (iNOS) includes nitric oxide and peroxynitrite. These reactive oxygen species (ROS) functions as cellular messengers [11], regulator of apoptotic process of neutrophils [12], modulator of reactive immune cells [13] and they have been implicated as crucial in the control of Mtb infection or subsequent progression to active disease. Modest increases in the levels of these reactive species at site of local infection substantially damage DNA, lipids and proteins, particularly iron-sulfur (4Fe-4S) cluster proteins, in diverse bacteria including Mtb [14,15] thereby eliciting bacteriostatic and bactericidal effects. However, the reasons why immune competent Mtb infected individuals develop active disease in the presence of these potent antimicrobial mechanisms is not clearly understood.

This study therefore assessed components of respiratory burst pathway and nitric oxide in sputum and lysate of PBMCs from Nigerian TB patients before commencement of anti-TB chemotherapy. The aim of this study is to identify which aspect of cellular innate immune response can be explored as immunotherapeutic target.

Participants and Methods

A total of 160 participants were enrolled for this study. This comprised of fifty (50) multi-drug resistant TB (MDR-TB) patients, sixty (60) drug-sensitive TB (DS-TB) patients and fifty (50) non-TB apparently healthy individuals. MDR-TB patients had been previously diagnosed as being infected with isoniazid and rifampicin resistant strains of Mtb using clinical history, Chest X-ray and GENE Xpert. These patients were admitted into the MDR-TB centre, University College Hospital (UCH) Ibadan, Nigeria for anti-TB treatment. DS-TB patients were recruited from the Medicine Out-patient Clinic, University College Hospital, Ibadan, Nigeria by a Consultant Chest Physician after confirmation with Microbiological test (sputum smear microscopy), chest X-ray and clinical history.

Five milliliters (5 ml) of blood was drawn from the antecubital vein of each participant and dispensed into lithium heparin containing sample tubes. Blood was diluted with equal volume of phosphate buffered saline (PBS). Diluted blood was carefully overlaid onto Ficoll to avoid disturbance of the Ficoll layer, in the ratio 2:1. Mixture was centrifuged at 1000g for 15mins and PBMCs were obtained. PBMCs obtained were washed, resuspended in Ringers solution and PBMCs lysate was obtained freeze thaw method as described [16,17]. Cell suspension was frozen for 15 minutes at -20°C and thawed at 4°C for 30 minutes. This procedure of freezing (-20°C, 15 minutes) and thawing (4°C, 30 minutes) was repeated to make three cycles. Microscopic examination confirmed complete disruption of PBMCs. Lysate was stored at -20°C until analysis.

Spot collection of sputum sample was carried out. Study participants were asked to rinse their mouth with water to reduce salivary contamination of sputum. Sputum produced was collected into 20 ml sterile disposable polypropylene tubes and kept on ice from collection to arrival at the laboratory. Sputum sample was homogenized with an equal volume of phosphate buffered saline (PBS). Homogenate was centrifuged at 10,000g for 10 minutes to remove cellular debris and mucus, and supernatant obtained was stored at -20°C until analysis.

Superoxide Dismutase (SOD) activity determination

The SOD activity was determined using the method of Misra and Fridovich [18] as previously carried out [19]. This method is based on the principle that SOD inhibits the autoxidation of epinephrine at pH 10.2.

Catalase (CAT) activity determination

Catalase activity was determined using the method of Sinha [20] as previously carried out [19]. This method is based on the principle that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured at 570 nm.

Myeloperoxidase (MPO) activity determination

MPO activity was determined using the method of Desser., *et al* [21]. The rate of decomposition of H_2O_2 by peroxidase, with guaiacol as hydrogen donor, produced tetraguaiacol which was measured at 436 nm and at 25^{0}C.

Hydrogen peroxide determination

Hydrogen peroxide concentration was determined as described by Wolff [22] and previously carried out [19]. The assay is based on peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange to form Fe^{3+}-xylenol orange complex with an absorbance maximum of 560 nm. Plasma H_2O_2 was determined by comparing absorbance with standard solutions of H_2O_2

Nitric oxide (NO) determination

Nitric oxide concentration in sputum and PBMCs lysate was determined using Griess reagent (Sulpanilamide and N-1-napthyethylenediamine dihydrochloride) as previously described [23]. The assay is based on a reaction that utilizes sulpanilamide and N-1-napthyethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. Nitrite forms coloured chromophore with reagent, with an absorbance maximum at 540nm. The production of nitrite is quantified by comparing the result with absorbances of standard solutions of sodium nitrite.

Result

Sputum SOD activity was significantly increased in MDR-TB patients, though significantly decreased in DS-TB patients compared with controls (Table 1). There were significant increases in sputum H_2O_2 level and sputum MPO activity in both MDR-TB and DS-TB patients compared with controls. Sputum NO level was also significantly increased in MDR-TB patients compared with controls (Table 1). MDR-TB patients had significantly increased SOD activity, H_2O_2 and NO levels compared with DS-TB patients (Table 1).

Variables	Sample	MDR - TB	DS - TB	Controls	p
SOD	Sputum	0.56 (0.43 - 0.66)[a,b]	0.17 (0.10 - 0.18)[a]	0.19 (0.17 - 0.20)	0.000*
	PBMCs lysate	0.48 (0.46 - 0.52)[b]	0.18 (0.16 - 0.47)[a]	0.49 (0.40 - 0.68)	0.000*
CAT	Sputum	36.63 (19.49 - 59.73)	27.81 (14.6 - 90.2)	36.37 (26.5 - 77.6)	0.810
	PBMCs lysate	115.3 (76.6 - 139.8)	124.2 (73.8 - 141.3)	115.6 (70.9 - 140.8)	0.719
MPO	Sputum	2.34 (1.40 - 4.01)[a]	5.26 (1.26 - 13.06)[a]	0.46 (0.07 - 0.89)	0.000*
	PBMCs lysate	1.99 (1.67 - 4.38)[b]	4.09 (2.43 - 4.66)	3.12 (1.94 - 7.59)	0.096
H_2O_2	Sputum	268.4 (165.4 - 407.4)[a,b]	130.7 (119.9 - 138.6)[a]	123.6 (113.6 - 140.6)	0.000*
	PBMCs lysate	20.05 (18.0 - 21.7)[b]	29.4 (27.33 - 30.39)	23.49 (19.75 - 33.87)	0.000*
NO	Sputum	76.5 (52.8 - 119.9)[a,b]	17.9 (10.9 - 25.8)	7.3 (2.9 - 22.7)	0.000*
	PBMCs lysate	4.56 (3.38 - 5.33)[a]	6.06 (2.28 - 6.79)	6.78 (4.28 - 10.74)	0.039*

Table 1: *Comparison of respiratory burst enzymes activity, levels of hydrogen peroxide and nitric oxide in sputum and PBMCs lysate of pulmonary tuberculosis patients at diagnosis with non-tuberculosis controls.*

**Significant at $p < 0.05$*

[a]: Significantly different from Control

[b]: Significantly different from DS - TB

There were significant decreases in SOD activity and NO levels in PBMCs lysate of DS-TB and MDR-TB patients respectively when compared to controls. Though SOD activity was significantly raised, there were significant decreases in lysate H_2O_2, NO and MPO activity in MDR-TB patients when compared with DS-TB patients (Table 1).

Discussion

This study is an extension of our earlier study which reported no difference in intracellular killing of leukocytes from TB patients at diagnosis compared with controls [23]. The phagocyte respiratory burst is important for controlling infections caused by many pathogens, as evidenced by clinical observations in patients with chronic granulomatous disease [24]. The role of ROS in anti-mycobacterial immunity has been highlighted by the discovery of a mutation in the gene encoding the catalytic subunit $gp91^{phox}$ of NADPH oxidase 2 (NOX2) linked to TB susceptibility in patients [25]. The generation of ROS requires assembly of the superoxide-generating NADPH oxidase 2 (NOX2) complex at phagolysosomal membranes [26] that catalyzes the reduction of molecular oxygen to superoxide (O_2^-). Superoxide dismutase (SOD) catalyzes superoxide conversion to H_2O_2, another potent antimicrobial agent. In this present study, sputum SOD activity in MDR-TB patients was raised where as in DS-TB patients sputum and PBMCs lysate SOD activity were reduced compared with controls. This might be due to differences in the strain of Mtb causing DS-TB and MDR-TB as previously reported by Romero., *et al.* [27] who demonstrated differences in innate immune responses elicited by different Mtb strains. This result can also be attributed to raised plasma zinc

(Zn) and copper (Cu) in MDR-TB compared with DS-TB previously reported by us [28,29]. Zinc and copper ions are the catalytic center of SOD thus changes in the levels of Zn and Cu will also affect the activity of SOD.

In this present study, sputum H_2O_2 increased significantly in MDR-TB and DS-TB patients when compared with controls while sputum NO increased in MDR-TB patients compared with controls. Our finding of increased sputum H_2O_2 in TB patients agrees with previous report of elevated exhalation of H_2O_2 in patients with pulmonary tuberculosis [30]. A previous study indicated that H_2O_2 acts as a macrophage-activating factor by augmenting the release of tumor necrosis factor-α [31] and promoting cell death by apoptosis. Phagocytic cell death by apoptosis restricts Mtb growth during the early phase of infection and also plays an important role in induction of the acquired cellular immune response [32]. It might be conceived that raised H_2O_2 is an innate immune component of Mtb control.

Increased sputum H_2O_2 and NO observed in this study is indicative of local lung inflammation which may be exploited by Mtb to promote necrotic cell death and other cascade of events that are characteristic of TB reactivation. Studies have implicated excessive inflammation in tuberculosis susceptibility and progression [33-35]. Therapeutic potential of ROS scavengers against TB has been demonstrated in zebrafish [36]. However, studies in humans are required to fully understand the nature of these interactions so as to identify specific therapeutic targets. The enzyme myeloperoxidase catalyzes the production of hypochlorous acid, another potent microbicidal agent. Sputum myeloperoxidase activity was increased in TB patients compared with controls in this present study. This further shows that indices of phagocytosis are not impaired in TB patients. A previous study demonstrated that Mtb-induced necrosis depends on NADPH–oxidase/myeloperoxidase function [37].

Despite raised levels of mediators of phagocytosis in TB patients, yet Mtb subverts host immune defenses. It is thus hypothesized that Mtb mechanisms of evading host immunity supersedes host protective immune responses. Mycobacterial superoxide dismutase (Sod A and Sod C), catalase-peroxidase (Kat G) and the alkyl hydroperoxide reductase have been shown to confer resistance on Mtb from damage by ROS and RNS [38,39].

Conclusion

In conclusion, the significantly raised levels of ROI and RNS in the sputum of Mtb patients might be a mechanism in the local environment of the host to prevent spread or progression of Mtb. However, It may be conjectured from the result of this study and our previous publication [23] that the progression of Mtb infection to active disease despite raised levels of ROS and RNS in TB patients indicate effective ROS/RNS evading mechanisms of Mtb.

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Conflict of Interest

None declared.

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