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ORIGINAL RESEARCH

Innate Cellular Immunity in Newly Diagnosed Pulmonary Tuberculosis Patients and During Chemotherapy

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Abstract

BACKGROUND Leukocyte migration (LM) and intracellular killing aspects of the innate immune response play important roles in protection against and containment and cure of *Mycobacterium tuberculosis* infection, and thus may be exploited as immunotherapeutic targets to improve the management and treatment outcomes of patients with tuberculosis (TB).

OBJECTIVES The aim of this study was to assess LM and mediators of intracellular killing in patients with TB at the time of diagnosis and during anti-TB chemotherapy and compare them with apparently healthy controls.

METHODS We recruited 24 patients who were newly diagnosed with pulmonary TB and 20 apparently healthy individuals. Blood was drawn from patients with TB at the time of diagnosis, and after 2, 4, and 6 months of anti-TB chemotherapy and control. In vitro percentage LM (%LM) upon stimulation with Bacillus Calmette-Guérin vaccine, percentage nitroblue tetrazolium (%NBT) reduction, plasma concentrations of hydrogen peroxide (H_2O_2), and nitric oxide (NO) were assessed in both groups.

FINDINGS Percentage NBT was significantly reduced in patients with TB at 2 months of anti-TB chemotherapy compared with patients at diagnosis and in healthy controls, whereas %LM was significantly increased in patients at 4 months of anti-TB chemotherapy compared with patients at diagnosis and controls. Mean plasma H_2O_2 and NO were significantly reduced in patients at diagnosis and throughout the period of anti-TB chemotherapy compared with the control group. Significant decreases were demonstrated in mean plasma H_2O_2 and NO in patients at 2 and 4 months of anti-TB chemotherapy, respectively, compared with patients at diagnosis. There was significant positive correlation between %NBT with plasma H_2O_2 and NO, but %LM was negatively correlated with plasma H_2O_2 in this group.

CONCLUSION The intracellular killing aspect of innate cellular immunity is deficient in patients with TB, especially 2 to 4 months after commencement of treatment. Therefore, measures (eg, arginine supplementation) to improve intracellular killing in these patients is advocated. Moreover, %LM assay with Bacillus Calmette-Guérin vaccine as an antigen may be used to differentiate those newly diagnosed patients from those on anti-TB chemotherapy.

KEY WORDS anti-TB chemotherapy, intracellular killing, leukocyte migration, nitroblue tretrazolium, pulmonary tuberculosis

The authors state that they have no conflicts of interest. All authors had access to the data presented in this manuscript and had a role in writing the manuscript.

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INTRODUCTION

Death from tuberculosis (TB) is increasing as a result of poverty, malnutrition, synergy with the human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) pandemic, and the emergence of multidrug- and extensively drug-resistant strains of *Mycobacterium tuberculosis*.[1] The host immune system is a critical factor for containment and cure of *M. tuberculosis* (Mtb) infection, whereas immunotherapy as an adjunct to drug treatment has the potential to improve treatment outcome.[2,3] Hence, there is a need for a clear understanding of immune response to Mtb infection to propose an efficient adjuvant immunotherapeutic agent or mechanism.

The innate immune response plays an important role in protection against Mtb. After MtB enters the alveolar space via inhalation, it is phagocytosed by alveolar resident macrophages and dendritic cells as an innate immune response to contain and eradicate the bacilli.[4] This leads to a rapid inflammatory response and recruitment of other immune cells to the lungs. However, the bacteria use different mechanisms to evade elimination, thereby exploiting the macrophage as a niche for growth and expansion.[4]

During infection, polymorphonuclear phagocytes use oxygen-dependent and oxygen-independent mechanisms in an attempt to eliminate invading pathogens.[5] The oxygen-dependent mechanism involves the production of reactive oxygen intermediates (ROI) such as superoxide radical, hydroxyl radical, hydrogen peroxide (H_2O_2), and hypochlorous acid in the respiratory burst pathway as well as production of reactive nitrogen intermediates such as nitric oxide (NO) and peroxynitrite via the inducible nitric oxide synthase. Although phagocytes have been implicated in the control of mycobacterial infections,[6,7] the mechanisms by which they are attracted to the site of infection as well as the mechanisms by which they exert protective function are not clearly resolved.

This study, therefore, assessed 2 aspects of phagocytosis (leukocyte migration [LM] and mechanisms of intracellular killing) in Nigerian patients with TB at the time of diagnosis and throughout antituberculosis (anti-TB) chemotherapy. In vitro percent migration of leukocytes was determined after stimulation with Bacillus Calmette-Guérin (BCG) vaccine, whereas intracellular killing mechanism was determined by evaluating plasma levels of NO, H_2O_2, and leukocyte nitroblue tetrazolium (NBT) dye reduction tests. The aim of the present study was to identify which aspect of cellular innate immune response should be used as immunotherapeutic target.

PARTICIPANTS AND METHODS

We recruited 44 participants for the study, which comprised 24 patients with newly diagnosed pulmonary tuberculosis and 20 apparently healthy individuals without pulmonary tuberculosis after obtaining written informed consent. Participants with HIV and helminthes infection were excluded, as were patients on any medication or those who were pregnant. The study protocol was reviewed and approved by the University of Ibadan/University College Hospital Joint Institutional Research Ethics Committee. All patients were recruited from the Medicine Outpatient Clinics, University College Hospital, Ibadan, Nigeria, by a consultant chest physician after laboratory tests, chest x-rays, and clinical history.

At diagnosis of TB, 5 mL of blood was withdrawn from the antecubital fosa vein into lithium heparin tubes at diagnosis; blood was drawn again at 2, 4, and 6 months (completion of duration) of anti-TB therapy.

Percentage Leukocyte Migration. Percentage leukocyte migration (%LM) was determined as previously described.[8] Leukocytes were isolated from whole blood using 6% dextran. After separation of plasma by centrifugation, 6% dextran was mixed with packed cells (1:1) and incubated for 45 minutes at 37°C. Leukocyte-rich supernatant was obtained and washed 3 times in Kreb-Ringers solution, filled into capillary tubes, and anchored into a migration chamber filled with either Kreb-Ringers solution or antigen (BCG) and Kreb-Ringers solution (1:50). This was incubated for 18 hours at 37°C and the area of LM in the chamber containing antigen was compared with the area of migration in the chamber without antigen. The %LM was calculated as follows:

%LM = (area of migration in antigen solution/area of migration in medium alone) × 100.

Percentage LM value of 80% or less was considered positive.[8]

Percentage Nitroblue Tetrazolium Dye Reduction. Percentage nitroblue tetrazolium (%NBT) dye reduction was based on a previously described method.[8] For stimulated NBT procedure, 50 µL of NBT solution (0.2% NBT), 25 µL heparinized blood, and 25 µL of stimulant solution (nonviable bacterial extract) were mixed gently, incubated at 37°C for 10 minutes, and then incubated at 25°C

for 10 minutes. A thick smear of the mixture was prepared and allowed to air dry. Air-dried smear was treated with Wright stain for 15 seconds and flooded with distilled water for 30 seconds before rinsing in water and air-drying. Two hundred leukocytes were counted under an oil immersion objective and leukocytes showing dark formazan deposit were recorded as positive. The percentage of bacterially stimulated NBT was calculated as:

%NBT = (leukocyte with dark formazan deposit (positive)/total leukocytes counted) × 100.

Plasma NO Determination. Plasma was obtained from blood after centrifugation. NO concentration in plasma was determined using Griess reagent (Sulpanilamide and N-1-napthyethylenediamine dihydrochloride) as previously described.[9] The assay was based on a reaction that used sulpanilamide and N-1-napthyethylenediamine dihydrochloride under acidic (phosphoric acid) conditions. Nitrite formed colored chromophore with reagent, with an absorbance maximum at 540 nm. The production of nitrite was quantified by comparing the result with absorbances of standard solutions of sodium nitrite.

Plasma H_2O_2 Determination. Hydrogen peroxide concentration in plasma was determined as previously described.[10,11] This assay was based on the peroxide-mediated oxidation of iron $(Fe)^{2+}$, 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 curves of H_2O_2 obtained by eliminating H_2O_2 with catalase.

Statistical Analysis. The data obtained were analyzed using SPSS version 17.0. An independent Student's *t* test was used to compare mean values between the 2 groups. A paired *t* test was used to compare mean values of TB patients at diagnosis with values at 2, 4, and 6 months of anti-TB regimen. Pearson's correlation was used to test the correlation between variables in patients with TB and healthy controls. Values were considered significant at $P < 0.05$.

RESULTS

Percentage NBT was significantly reduced in patients with TB at 2 months of anti-TB chemotherapy compared with patients at diagnosis and the control group. Percentage LM was significantly increased in patients at 4 months of anti-TB chemotherapy compared with those at diagnosis and the control participants (Table 1).

Mean plasma concentrations of H_2O_2 and NO were significantly reduced in the patients at the time of diagnosis and throughout anti-TB chemotherapy compared with controls (Table 1). There also were significant decreases in mean plasma concentrations of H_2O_2 and NO in patients at 2 and 4 months of anti-TB chemotherapy, respectively, compared with patients at diagnosis (Table 1).

A significant positive correlation was noted between %NBT with plasma H_2O_2 and NO in the patients with TB, but %LM was negatively correlated with plasma H_2O_2 in these individuals (Table 2).

DISCUSSION

Leukocyte migration and oxygen-dependent intracellular killing are important processes in immunological resistance during infections. Phagocytes are attracted to the site of infection, activated, and retained at the site by cytokines and other serum factors. Attraction of phagocytes to the infectious agent leads to engulfment, phagolysosome formation, and intracellular killing of the ingested agent.

Table 1. %NBT, %LM, and Plasma Concentrations of H_2O_2 and NO in TB Patients at Diagnosis and During Anti-TB Therapy, and Healthy Controls

	At Diagnosis	Anti-TB Therapy			Healthy Controls
		2 mo	4 mo	6 mo	
%LM	56.72 ± 14.6	67.88 ± 25.5	75.34 ± 11.9*,†	60.26 ± 9.9	55.04 ± 14.9
%NBT	86.19 ± 10.4	72.32 ± 13.6*,†	80.53 ± 8.6	85.30 ± 3.9	83.33 ± 7.6
H_2O_2	9.70 ± 2.27*	7.59 ± 2.19*,†	7.96 ± 2.57*	9.58 ± 2.00*	12.68 ± 4.72
NO	7.40 ± 4.95*	5.44 ± 3.59*	3.40 ± 1.72*,†	6.57 ± 3.08*	10.86 ± 5.76

H_2O_2, hydrogen peroxide; NO, nitric oxide; %LM, percentage leukocyte migration; %NBT, nitroblue tetrazolium; TB, tuberculosis.
* Significant from controls ($P \leq 0.05$).
† Significant from diagnosis ($P \leq 0.05$).

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Table 2. Correlation of %NBT and %LM with Plasma H_2O_2 and NO in TB patients and Controls

	%NBT		%LM	
	DSTB Patients	Control	DSTB Patients	Control
H_2O_2				
r	0.406	0.385	−0.443	0.166
P	0.010*	0.216	0.005*	0.606
NO				
r	0.314	0.277	−0.199	−0.383
P	0.050*	0.383	0.225	0.219

DSTB, drug-susceptible tuberculosis patients; H_2O_2, hydrogen peroxide; %LM, percentage leukocyte migration; %NBT, nitroblue tetrazolium; NO, nitric oxide; TB, tuberculosis.
* Significant at $P \leq 0.05$.

In the present study, %LM with BCG antigen was found to be raised in patients with TB, especially at 4 months of anti-TB chemotherapy, compared with controls. Percentage LM is an in vitro method of assessing a delayed hypersensitivity state in which LM inhibitory factor (LMIF) secretion by lymphocytes inhibits free migration of leukocytes, thus resulting in reduced %LM.[12] Therefore, increased %LM in patients with TB at 4 months of anti-TB chemotherapy indicates that LMIF production was reduced in these individuals during anti-TB chemotherapy but not at diagnosis. BCG is a TB vaccine antigen administered to invoke protective immunity against Mtb infection in vaccinated hosts. In patients with TB, however, presensitization with Mtb bacteria would have stimulated the immune system of these infected patients to produce Mtb-specific cytokines including LMIF. Hence, in vitro BCG stimulation of presensitized leukocytes obtained from patients with TB would have resulted in increased production of LMIF to cause decreased %LM at diagnosis, as observed in the present study. However, during anti-TB chemotherapy, the Mtb bacteria load would have been reduced, thus reducing the number of Mtb presensitized cells and resulting in increased %LM-to-BCG stimulation in vitro. This implies that LMIF assay using BCG may be added to tools for monitoring efficacy of anti-TB chemotherapy.

The nonsignificant difference in %LM of control compared with %LM of TB patients at diagnosis may be due to previous exposure of controls to wild forms of Mtb leading to presensitization of controls. This reduces the usefulness of %LM with the BCG vaccine antigen as a screening tool for TB in the environment. This proposition supports the observation of positive Mantoux skin tests using purified protein derivative among healthy individuals in Nigeria,[13] thus necessitating the use of chest x-ray and other tests for confirmatory diagnosis of TB in this country. Increased %LM observed in the present study supports a previous report of decreased neutrophil chemotaxis in patients receiving anti-TB treatment.[14] Raised % LM (reduced LMIF production) observed in the present study also may be a mechanism to evade innate immunity by Mtb or an immune mechanism to control inflammation. Reduced %LM at diagnosis compared with %LM during anti-TB chemotherapy observed in the present study also may be an indication of increased susceptibility of patients with TB to other infections before treatment, as leukocytes may not be allowed to migrate freely to other infectious agents apart from Mtb. This was reversed with anti-TB chemotherapy. The use of nonmycobacteria antigen to stimulate leukocyte migration is suggested for future study.

Efficient phagocytosis and the subsequent production of ROI are important for intracellular killing of microorganisms by phagocytes. Defects in one or both of these functions may lead to deficient killing of Mtb or other opportunistic infections in patients with TB. The NBT dye reduction test is an in vitro measure of pathogen phagocytosis and ROI-generating activity of phagocytes.[15] Isolated leukocytes stimulated with an antigen produce ROIs, which reduce NBT to produce dark formazan deposit. It is believed that cells containing intracellular dark formazan deposits are involved in active ROI production via phagocytosis. Phagocytic cells increase consumption of oxygen after engulfment of infectious agents to produce ROIs (superoxide radical, H_2O_2, hydroxyl radical), which are used to kill infectious agents.[15] The present study found significantly reduced %NBT index in patients after 2 months of anti-TB chemotherapy compared with healthy controls and at patients diagnosis, consistent with previous reports.[16]

Results of the present study showing significantly reduced %NBT in TB patients, especially at 2 months postcommencement of anti-TB chemotherapy, indicate reduced production of ROI capable of destroying ingested pathogens, thus explaining continued existence of Mtb in macrophages and neutrophils, or innate cellular immunosuppression in this group of patients. This finding is supported by significant positive correlations of %NBT with plasma H_2O_2 as well as NO in patients

with TB. The present study also found reduced H_2O_2 and NO in patients with TB at diagnosis and during anti-TB chemotherapy compared with healthy controls. This justified the observed reduction in %NBT.

The ability of intracellular pathogens to influence inflammation and immune clearance by modulating cellular ROI previously has been reported.[17] In addition to its involvement in the direct killing of pathogens, ROI forms a key part of the intracellular redox profile, influencing a wide variety of signaling networks.[18] A recent study indicated that H_2O_2 released by activated neutrophils may act as a macrophage-activating factor by augmenting the release of tumor necrosis factor-α.[19] The present study found significantly reduced plasma H_2O_2 concentrations in patients at diagnosis and throughout the anti-TB treatment period compared with healthy controls. This finding is in contrast with a previous report that found higher plasma H_2O_2 in patients with TB compared with controls.[20] The difference might have been due to the inclusion of patients with HIV-TB coinfection in the group of TB patients in the study.[20]

Mycobacterial catalase-peroxidase protein and the alkyl hydroperoxide reductase protein have been shown to confer resistance on Mtb to the killing function of ROI.[21] Activity of these proteins also may have accounted for the reduced plasma H_2O_2 concentration observed at diagnosis and throughout the months of anti-TB chemotherapy. Reduced plasma H_2O_2 in these patients might account for their immunosuppression. Hydrogen peroxide is an ROI that is catabolized to produce hypochlorous acid and hydroxyl radicals. These highly reactive oxygen species have been reported to be effective in the killing of intracellular bacteria.[22] Thus, reduced H_2O_2 in patients with TB may be responsible for chronic survival of Mtb in the cells and susceptibility of these patients to other intracellular organisms. Hydrogen peroxide also has been reported to modulate expression of the leukocyte adhesion molecule and leukocyte endothelial adhesion.[23] The present study found a significantly negative correlation between plasma H_2O_2 and %LM in patients with TB. This implies an inverse relation between plasma H_2O_2 and %LM, which indicates that H_2O_2 has no effect on migration of leukocytes stimulated by BCG, although positive correlation was observed between H_2O_2 and NBT (an indicator of intracellular killing).

Interferon-γ–induced production of NO has been identified as a potent agent of Mtb immunity.[4] In addition to its role in intracellular killing of pathogens and vascular smooth muscle relaxation, NO modulates gene expression via the agency of transcription factors, with the most important being nuclear factor-κB (NF-κB), which regulates transcription of proinflammatory cytokines (interleukin [IL]-1β, tumor necrosis factor-α, IL-6, and IL-8) and enzyme,[24] which in turn are important in the control of bacterial growth. In the present study, plasma concentrations of NO in patients with TB at diagnosis and during anti-TB chemotherapy were significantly reduced compared with plasma NO in the control group. This is in agreement with a previous report[25] but is in contrast with two other studies.[26,27]

The reduction of NO observed in the present study is indicative of reduced intracellular killing of Mtb and innate cellular immunosuppression in patients with TB, which may account partly for the continuous persistence of Mtb in macrophages and neutrophils. NO is produced by converting arginine, an amino acid, into citrulline using inducible nitric oxide synthase in phagocytes. Micronutrient malnutrition is a significant feature in Mtb patients[28,29]; thus, it is likely that arginine might be reduced in the group of patients with TB considered in the present study. This suggestion is supported by observation that supplementation of arginine during anti-TB chemotherapy improved clinical outcome in patients with TB.[30] Arginine supplementation as an adjuvant to anti-TB chemotherapy in the treatment of these patients is therefore recommended for Nigerian patients with TB.

CONCLUSION

The intracellular killing aspect of innate cellular immunity is deficient in patients with TB, especially 2 to 4 months after commencement of treatment. As such, measures (such as arginine supplementation) to improve intracellular killing in these individuals is advocated. Moreover, %LM assay with BCG vaccine as antigen may be used to differentiate patients newly diagnosed with TB from those already on anti-TB chemotherapy.

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