HOST RESPONSE TO INFECTION

NADH-oxidase, NADPH-oxidase and myeloperoxidase activity of visceral leishmaniasis patients

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It is believed that the enhanced capability of activated macrophages to resist infection is related to the remarkable increase in the production of oxygen metabolites in response to phagocytosis. Both the production of H_2O_2 and the oxidation of NAD(P)H are directly dependent upon NAD(P)H-oxidase. It has been established that the respiratory burst is due to activation of NAD(P)H-oxidase localised in the plasmalemma. Myeloperoxidase is believed to be involved in augmenting the cytotoxic activity of H_2O_2 . Low NADH-oxidase, NADPH-oxidase and myeloperoxidase activity were observed in monocytes of patients with active visceral leishmaniasis as compared with healthy controls. These results suggest that low NADH-oxidase, NADPH-oxidase and myeloperoxidase activities may account for persistence of *Leishmania* parasites in visceral leishmaniasis.

Introduction

Visceral leishmaniasis (VL) or kala-azar is caused by *Leishmania donovani*, an obligate intracellular protozoan that parasitises tissue macrophages. Intramacrophage infection by *L. donovani* results in potentially fatal visceral infections in man and the elimination of *Leishmania* parasites by the macrophage depends upon the mounting of an effective cell-mediated immune response by the mammalian host [1].

The diseases caused by all species of the *Leishmania* genus are determined by the fact that these parasites multiply and survive in the microbicidal environment of mononuclear phagocytes [2]. This survival has to be seen in the context that promastigotes of *Leishmania* are destroyed *in vitro* by the metabolites of the oxidative burst, hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) [3] with H_2O_2 being more effective than O_2^- . The respiratory burst is due to increased activation of NAD(P)H-oxidase localised in the plasmalemma [4]. The generation of reactive oxidative species (ROIs) is associated with production of certain oxygen metabolites, which is linked to induction of cellular damage, microbicidal activity and the regula-

tion of the activity of natural killer cells, polymorphonuclear leucocytes (PMNLs) and monocytes [5]. The conversion of oxygen to microbicidal product is mediated by a plasma membrane-associated enzymes or enzyme system.

Myeloperoxidase (MPO; H_2O_2 oxidoreductase) is an enzyme found in the azurophilic granules of mammalian neutrophils and also identified in human monocytes. PMNLs employ a system comprised of MPO, H_2O_2 and oxidisable halide co-factor to kill a variety of micro-organisms [6]. MPO is believed to be involved in augmenting the cytotoxic activity of H_2O_2 and O_2^- . Klebenoff [6] has demonstrated that, during endocytosis, MPO is released into the phagosomes as a result of fusion of the phagosome membrane with the azurophilic granules. Extracellular release of granule components occurs upon contact of the PMNLs with a large membrane or membrane-like surface. Therefore, NADH-oxidase, NADPH-oxidase and MPO activity were examined in monocytes from patients with active VL.

Materials and methods

Reagents

All reagents were prepared in quartz distilled water. Ficoll-Hypaque (Lymphoprep) was obtained from Nycomed Pharma, Norway. RPMI 1640, Hanks's

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Balanced Salts Solution (HBSS), fetal calf serum (FCS) and other reagents were purchased from Sigma. Culture plates were purchased from A/S Nunc, Denmark. NADH, NAD(P)H, SDS, OPD, DMSO and H_2O_2 were purchased from Hi Media, India.

Blood samples

Blood samples of 15 patients with active VL and five endemic area controls were collected from the Kala-azar Medical Research Centre, Muzaffarpur, Bihar. Blood samples of five healthy controls from non-endemic areas were collected from the Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh.

Isolation of monocytes from peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood by the Ficoll-Hypaque density gradient method [7] with centrifugation at 2200 rpm for 25 min. PBMC were resuspended in RPMI-1640 supplemented with heat-inactivated FCS 10%, gentamicin 20 mg/L, streptomycin 100 mg/L and penicillin 100 mg/L. Monocytes (2×10^5 cells/well) were obtained by adherence for 2 h at 37°C in tissue-culture plates in a CO_2 incubator (CO_2 5%, humidity 95%).

Assay for NADH- and NADPH-oxidase [8]

Monocytes were washed with fresh culture medium and the cells were lysed in 2.0 ml of SDS 0.25% in distilled water; 0.5 ml of this cell lysate was added to a tube containing 2.5 ml of 0.1 M sodium phosphate buffer, pH 7.5. The OD_{340} of the sample was determined for NADPH- and NADH-oxidase activity. For preparing standards, accurately weighed amounts of β -nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NADH) (2 mg of each) were dissolved in 5 ml of phosphate buffer, pH 7.5. The OD_{340} of each of several dilutions of this stock solution was read against the buffer.

A calibration curve was plotted and the OD_{340} values of samples were compared with the standard OD_{340} of the NADH-oxidase and NADPH-oxidase. The increase or decrease in the OD_{340} was directly related to the increase or decrease in the enzyme activity. The data are represented as increase or decrease in OD_{340} .

Assay for MPO

MPO activity of monocytes was assessed in the cell lysates by the following method [8]. Monocytes were lysed in Triton X-100 0.05% and were kept frozen at -70°C until use. The substrate cocktail contained 5 ml of 0.1 M citrate buffer (pH 5.5), 32 μl of Triton X-100 20%, 50 μl of 82.4 mM *O*-Dianisidine or OPD in

dimethyl sulphoxide (20.1 mg/ml in DMSO) and 20 μl of 26.4 mM H_2O_2 . To 0.5 ml of cell lysate, 0.7 ml of 0.1 M citrate buffer (pH 5.5) was added. Then 4.8 ml of the above assay mixture was again added. The mixture was kept at room temperature for 1 h and the OD_{450} was measured.

Enzyme activity at room temperature =

$$\frac{\text{OD}_{450}^{\text{E}} - \text{OD}_{450}^{\text{B}} \text{ units/min/ml of cell lysate}}{\text{time (min)} \times \text{volume of samples (ml)}}$$

where $\text{OD}_{450}^{\text{E}}$ = OD_{450} of experimental sample and $\text{OD}_{450}^{\text{B}}$ = OD_{450} of blank.

Results

Study population

The clinical and laboratory features of the 15 patients on admission are summarised in Table 1.

NADH-oxidase activity in monocytes

Monocyte lysates were assayed for their NADH-oxidase activity against the standards. The increase or decrease in OD_{340} directly shows increased or decreased NADH-oxidase activity. Monocytes from healthy controls from endemic and non-endemic regions showed significantly greater NADH-oxidase activity than those from patients with active VL (Table 2).

NADPH-oxidase activity in monocytes

Monocyte lysates were assayed for their NADPH-oxidase activity against the standards. The increase or decrease in OD_{340} directly shows increased or decreased NADPH-oxidase activity. Monocytes from healthy controls from endemic and non-endemic regions showed significantly higher NADPH-oxidase activity than those from patients with active VL (Table 2).

Myeloperoxidase activity in monocytes

Monocytes isolated from healthy endemic and non-endemic controls showed significantly higher myeloperoxidase activity than those from active VL patients (Table 2).

Discussion

It is believed that the enhanced capability of activated macrophages to resist infection [9] is related to the remarkable increase in the production of oxygen metabolites in response to phagocytosis. It is further believed that the greater oxidative metabolic responses of the activated macrophages could play a marked role in the accelerated capacity to affect cell-mediated immunity.

Table 1. Clinical and laboratory features of patients with visceral leishmaniasis

Criteria	VL patients	Controls from	
		endemic region	non-endemic region
Number of patients	15	5	5
Age (years)	21.1 (3.8)*	25.6 (3.1)	27.2 (3.4)
Duration of illness (months)	4.3 (0.3)	...	...
Weight (kg)	43.3 (5.7)	52.8 (5.9)	59.0 (5.5)
Karnofsky score [†]	74.0 (1.6)	...	...
Spleen size (cm)	8.3 (1.3)	...	...
Splenic aspirate score	2.2 (0.3)	...	...
WBC count ($\times 10^3/\text{mm}^3$)	3.6 (0.88)	9.1 (1.1)	8.6 (1.2)
Haemoglobin (g/dL)	8.9 (0.5)	13.6 (1.5)	14.1 (1.7)
Platelet count ($\times 10^3/\text{mm}^3$)	0.7 (0.1)	175.0 (20.9)	184.0 (21.1)
*Results are means and (SD).			
[†] Karnofsky performance scale:			
Able to carry on normal activity: no special care is needed.	100	Normal, no complaints, no evidence of disease.	
	90	Able to carry on normal activity, minor signs or symptoms of disease.	
	80	Normal activity with effort, some signs or symptoms of disease.	
Unable to work, able to live at home and care for most personal needs, a varying amount of assistance is needed.	70	Unable to carry on normal activity or to do active work.	

Table 2. In-vitro NADH-oxidase, NADPH-oxidase and MPO activities in monocytes from patients with active VL and healthy controls

Disease status (n)	Mean (SD) NADH-oxidase activity (OD ₃₄₀)	Mean (SD) NADPH-oxidase activity (OD ₃₄₀)	Mean (SD) MPO activity (units/min/ml)
Acute VL patients (15)	7.86 (0.632)*	10.66 (0.665)**	$0.758 (0.047) \times 10^{-4}$ **
Uninfected (10)	9.15 (0.105)	12.33 (0.359)	$1.23 (0.042) \times 10^{-4}$

*Significantly decreased *versus* uninfected controls, $p < 0.01$.**Significantly decreased *over* uninfected controls, $p < 0.001$.

In the present study, decreased NADH-oxidase and NADPH-oxidase activity was observed in monocytes of patients with active VL than in controls. It can be suggested that down-regulated activity of NADH-oxidase and NADPH-oxidase may account for the persistence of *Leishmania* parasites in patients. As ROIs, a result of respiratory burst activity, are essential for anti-leishmanial activity by macrophages, both the production of H_2O_2 and oxidation of NADPH are directly dependent upon NADPH-oxidase [10].

In an earlier study, H_2O_2 , O_2^- and $\text{IFN-}\gamma$ production by monocytes from VL patients were examined [11]. H_2O_2 , O_2^- and $\text{IFN-}\gamma$ production by cultured monocytes from patients with active VL were significantly lower than in healthy controls. In contrast, nitrite levels in the supernates of monocyte cultures of VL patients were similar to those of healthy controls and increased significantly during antileishmanial therapy. On day 20 of treatment, a significant increase in the release of H_2O_2 , O_2^- and $\text{IFN-}\gamma$ was observed. However, at follow-up, 4 months after the end of treatment, the

production H_2O_2 , O_2^- , $\text{IFN-}\gamma$ and nitrite had declined significantly. Thus, the impairment in H_2O_2 and O_2^- production suggests that down-regulation of these mediators may be involved in the reduced killing of parasites by monocytes of patients with active VL. Furthermore, it suggests that an immune-based mechanism is involved in successful drug therapy [11].

It has been established that the respiratory burst is due to activation of NAD(P)H-oxidase localised in the plasmalemma. The formation of O_2^- radicals as well as other active forms of oxygen represents a major anti-infection defence by phagocytosing cells. The failure of phagocytes from patients with chronic granulomatous disease (CGD) to kill intracellular amastigotes even after treatment with $\text{IFN-}\gamma$ indicates the important role of the oxidative burst for this function [12]. Murray and Nathan [13] determined the relative contribution of ROIs *versus* reactive nitrogen intermediates (RNI) in respiratory burst-deficient gp91(phox)l-X-linked CGD and concluded that (a) ROI and RNI probably act together in the early stages

of infection and (b) RNI alone are necessary and sufficient for the eventual control of VL infection. The same cannot be said in the case of man, as scant information is available on role(s) of NO in the pathology of VL.

The role of ROIs in the phagocytic functions of Kupffer cells (KCs) and *Candida albicans* activity was studied by Potoka *et al.* [14]. Their study indicated that ROIs generated by both NADPH-oxidase- and xanthine oxidase-dependent pathways are important in killing of *C. parapsilosis* by KCs [14]. The neutrophil respiratory burst oxidase is a multi-component activatable enzyme comprising one of the major phagocyte antimicrobial systems. In the genetic disorder CGD, absent oxidase function is associated with recurrent, severe and often life-threatening infections [15].

Fazal [16] determined the role of ROIs in limiting the growth of intracellular mycobacteria within peripheral blood-derived neutrophils and monocyte-derived macrophages from patients with CGD and normal healthy human volunteers. CGD patients are known to have a hereditary defect in the NADPH-oxidase pathway, resulting in their neutrophils and monocyte-derived macrophages being unable to generate oxygen radicals which are required to kill intracellular bacteria [17]. The cells were infected with Bacille Calmette-Guérin (BCG) or *Mycobacterium avium*, and the bacterial growth in each cell type was determined by cfu estimate. The results indicated that there was no demonstrable inhibition in the intracellular mycobacterial growth within neutrophils or macrophages from either CGD patients (deficient in NADPH-oxidase pathway) or normal healthy volunteers. Macrophage treatment with either IFN- γ or TNF- α had no effect [16].

Condino-Neto *et al.* [18] investigated the NADPH-oxidase activity, cytochrome b558 content and gene expression of gp91-phox and p47-phox in normal Epstein-Barr virus (EBV)-transformed B lymphocytes and compared them to EBV-transformed B lymphocytes from patients with X-linked CGD, normal peripheral blood neutrophils or mononuclear cells, and the A301 or C8166 lymphoblastoid cell lines. They concluded that NADPH-oxidase activity and cytochrome b558 content correlate with gp91-phox and p47-phox gene expression in EBV-transformed B lymphocytes.

MPO is believed to be involved in augmenting the cytotoxic activity of H_2O_2 and O_2^- . H_2O_2 or other oxygen intermediates may mediate the toxic effects of macrophages, either directly or in combination with MPO. Low MPO activity observed in patients with active VL may contribute to survival of parasites in macrophages. It is well known that one of the biological functions of certain peroxidases is to form a potent cytotoxic compound. For MPO to act in this

capacity, i.e., to augment the cytotoxic activity of H_2O_2 and O_2^- , the MPO would have to have increased activity for target cell killing [19].

Rosen and Michel [20] found that neutrophils with an intact MPO antimicrobial system (normal or appropriately supplemented deficient cells) were capable of rapidly suppressing *Escherichia coli* DNA synthesis, whereas unsupplemented CGD or MPO-deficient cells were far less effective, indicating that the MPO system was active in normal neutrophils. The degree of DNA synthesis inhibition by MPO-sufficient neutrophils could account, in a cell-free system, for most of the observed microbicidal activity. Rapid and extensive inhibition of bacterial DNA synthesis appears to be an indicator of MPO activity in neutrophils [20].

MPO catalyses the reaction of H_2O_2 with chloride ion to produce hypochlorous acid (HOCl), which is used for microbial killing by phagocytic cells. Despite the important role of MPO in host defence, MPO deficiency is relatively common in man, and most of these individuals are in good health. Aratani *et al.* studied mice with no MPO activity in their neutrophils and monocytes. They developed normally, were fertile and showed normal clearance of intraperitoneal *Staphylococcus aureus*. However, they showed increased susceptibility to pneumonia and death following intratracheal infection with *C. albicans*. Furthermore, the lack of MPO significantly enhanced the dissemination of *C. albicans* injected intraperitoneally, thereby suggesting that MPO is important for early host defence against fungal infection, and the inability to generate HOCl cannot be compensated for by other oxygen-dependent systems *in vivo* in mice. The mutant mice serve as a model for studying pulmonary and systemic candidiasis [21].

The observation that this system is able to generate large IgG aggregates [22] suggests that it could be linked to the activation of phagocytic cells. Duc Dodon *et al.* [23] reported that hyposialylated IgG, either as monomers or as aggregates, can induce the decrease of intracellular MPO content in human monocytes. Indeed, all the visceralising species of *Leishmania* are known to stimulate the immunoglobulin-producing B cells non-specifically to produce antibodies of all classes and subtypes including IgA, IgM, IgG, etc. In contrast, albumin production is reduced and thus the albumin:globulin ratio is reversed and hypergammaglobinaemia is observed [24,25].

While there are some facts known, most of the theories regarding the immunopathogenesis of VL rely on inferences from human cutaneous leishmaniasis and clues obtained from murine models. No model mimics the various forms of human disease perfectly. However, there are no studies available on the biochemical aspects of respiratory burst activity in VL. To our knowledge, this is the first study that has investigated

NADH-oxidase, NADPH-oxidase and MPO activity in patients with active VL infections. We conclude that there is a down-regulation of NADH-oxidase, NADPH-oxidase and MPO (biochemical parameters of macrophage activation) activity in the pathology of VL infection.

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