Studies on the arginase, 5'-nucleotidase and lysozyme activity by monocytes from visceral leishmaniasis patients

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Abstract Intracellular pathogenic protozoan infection like visceral leishmaniasis is considered in terms of the overall inflammatory response and the complex cellular interactions leading to formation of the activated macrophage. Analysis of the development of activation is facilitated when operationally defined stage of activation are characterized using a library of objective markers. There is a role of arginase in the immune response supporting its involvement in macrophage effector mechanism in vitro and in vivo. 5'-Nucleotidase a plasma membrane component has been cited as a biochemical correlate of macrophage function in an altered morphological and biochemical state of activation and stimulation. Depression in 5'-nucleotidase activity has been generally referred to as a characteristic marker of activated macrophages. Lysozyme or lysosomal enzymes are released into the endocytic or autophagic vacuole macrophage where they serve the purpose of intracellular digestion of engulfed

or segregated materials. In the present study, we have studied levels of arginase and 5'-nucleotidase (marker for macrophage activation) in monocytes of active VL patients and healthy controls. Lysozyme a secretory product of macrophages was also measured in supernatants collected from monocytes of active VL patients and healthy controls. Elevated levels of 5'-nucleotidase were observed in supernatants of monocytes from active VL patients as compared to healthy controls. Low levels of arginase and lysozyme production by monocytes isolated from VL patients were observed as compared to healthy controls. Our studies suggest that low levels of arginase and elevated 5'-nucleotidase activity could be one of the mechanisms in the pathology of VL infection. Low lysozyme activity in patients may account for persistence of *Leishmania* parasites in VL infections.

Keywords Arginase · 5'-Nucleotidase ·
Lysozyme activity · Visceral leishmaniasis

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Introduction

Visceral leishmaniasis or kala-azar is caused by *Leishmania donovani* (Alder 1964; Pearson et al. 1983; Zuckerman and Lainson 1977). Kala-azar is endemic throughout many areas of South America, Africa, the Middle East and is resurgent in India and Bangladesh, where the disease was once thought to have been eradicated (Rahman and Islam 1983). These organisms infect exclusively macrophages of the mammalian hosts and subsequently live in the phagolysosomes of these cells (Xuan and Chang 1992). Intracellular parasites have evolved a different mechanism by which they are able to avoid the various soluble cytotoxic and or opsonizing products of the immune response. These organisms

sequester themselves behind the curtain of the host cell cytoplasmic membrane, a barrier that extra cellular, soluble immune products are not to breach. However, an intracellular milieu poses its own challenges to survival. Parasites must gain entry into the host cell. This sometimes requires activity entry (such as infection of erythrocytes or muscle cells) or they may take advantage of the cells own natural phagocytic activity (such as infection of macrophages). Additionally, although an anti parasite antibody response would have little effect on their survival; they must avoid eliciting a cellular immune response that would results in activated macrophage. In view of such myriad immune evasion mechanisms of parasites, one might wonder how it is ever possible for host immunity contain and resolve parasitic infections. Resolution of infection requires that the host generate an immune response with a cellular (T-cell) component such that activation of macrophages occurs. Analysis of the development of activation was facilitated when operationally defined stage of activation were characterized using a library of objective markers (Adams and Hamilton 1984).

Macrophages when activated by diverse stimuli synthesize and release a wide array of biologically active molecules, including several soluble suppressive factors (Allison 1978). One such product arginase has been demonstrated (Kung et al. 1977) in mixed lymphocyte culture (MLCS) done with an excess of peritoneal adherent cells. Visceral leishmaniasis is one of the scenario in which the activated mode of mononuclear phagocytes is found to be defective. In addition of digestion of exogenous molecules and particulates, macrophages are now known to modify their environment via the secretion of endogenously biosynthesized molecules that modulate inflammatory and immune response.

The term activation is commonly used to describe heightened states of macrophage function that are associated with an increased capacity of these cells to kill intracellular targets. There are several biochemical and structural correlate of macrophage activation.

For a long time investigations of arginase activity have been limited to the mammalian liver, where this enzyme is essentially involved in the urea cycle. The discovery of a substantial arginase production by activated macrophages has raised the question of its significance in the immune system. The release of arginase by activated macrophages depletes arginine from the extra cellular components resulting in inhibition of cell proliferation and in cytotoxicity.

5'-Nucleotidase a plasma membrane component has been cited as a biochemical criterion of macrophages in an altered morphological biochemical state of activation and stimulation (Edelson and Cohn 1976a, b; Karnovsky and Lazdins 1978; Pietrangeli et al. 1981). A decline in macrophages 5'-nucleotidase level is associated with activation

by *Coryne bacterium parvum* and BCG (Bruley-Rosset et al. 1976; Karnovsky and Lazdins 1978) and stimulation by sodium caseinate and thioglycollate (Edelson and Cohn 1976a, b). 5'-Nucleotidase hydrolyses the phospho ester linkage in 5'-mononucleotide liberating a nucleoside and inorganic phosphate. Werb and Cohn (Werb and Cohn 1972) reported that 5'-nucleotidase which is particularly rich in resident macrophage is lost progressively with activation.

Mononuclear phagocytes are also known for the production of lysozyme, a hydrolytic enzyme, which degrades and eliminates the microorganisms and is assessed by its ability to diminish the turbidity of suspension of killed *Micrococcus luteus*. Lysozyme (a mucopeptide *N*-acetylmuramyl hydrolase or muramidase) causes lysis of many Gram-positive bacteria by its action on their cell wall. Lysozyme is found in the granular fraction of mouse polymorph nuclear leucocytes, macrophages, human mononuclear phagocytes, lymphocytes and monocytes (Gordon et al. 1974). Lysozyme is a secretory product of macrophage, which is constitutively produced. Lysozyme and lysosomal enzymes are involved in the defense functions of the body.

In the present study, we have estimated levels of arginase, 5'-nucleotidase and lysozyme in the peripheral blood monocytes of VL patients to look at the state of macrophage activation as a consequence of Leishmania infection.

Materials and methods

Reagents

All reagents were prepared in quartz-distilled water. Ficoll-hypaque (Lymphoprep) was obtained from Nycomed Pharma, Norway. RPMI 1640, HBSS and Fetal calf serum (FCS) and other reagents were purchased from Sigma Chemical Co., MO, and USA. Culture plates were purchased from A/S Nunc, Denmark.

Patient samples

Peripheral blood samples of active patients ($n = 15$) and healthy controls ($n = 10$) were collected from Kala-azar Medical Research Centre, Muzaffarpur, Bihar and Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India.

Isolation of monocytes from peripheral blood mononuclear cells (PBMC)

Human PBMC were isolated from heparinized blood by Ficoll-hypaque density gradient centrifugation method (Boyum 1968) at 2200 rpm, for 25 min. PBMC were

resuspended in RPMI-1640 supplemented with 10% heat inactivated FCS, gentamycin (20 µg/ml), streptomycin (100 µg/ml) and penicillin (100 U/ml). The cells were counted in a Neuber's hemocytometer and viability was determined by trypan blue dye (Sigma Chemical Co. USA) exclusion method, of which 98% excluded trypan blue. Monocytes (2×10^6 cells/ml) were obtained by adherence for 2 h at 37°C in tissue culture plates in a CO₂ incubator (5% CO₂, 95% humidity). A positive control using LPS (10 µg/ml) was run in parallel with every assay.

Assay for arginase

Arginase activity was measured by the method described by Satoh and Ito (1968). Supernatants were collected from the monocytes (5×10^5 cells/ml) cultured for 2 h with medium alone or LPS. 0.5 ml of supernatant from monocyte cultures were added to centrifuge tubes, containing 0.5 ml of 0.424 uM arginine (pH 9.5) and 0.4 ml of 0.1 M Tris-buffer (pH 8.0). Reaction mixture was incubated at 37°C for 10 min. After 10 min, reaction were stopped by adding 1.0 ml of 87% acetic acid followed by the addition of 1.5 ml of 0.073 M of Ba(OH)₂ to remove amino acids and interfering materials as Barium salts. Then 0.4 ml of 0.273 M ZnSO₄ was added to this mixture and centrifuged at 1000 rpm for 10 min to separate the precipitate and to get clear supernatant. The clear supernatant was transferred to clean separately marked tube and urea content was estimated.

Estimation of urea

Five hundred micro liter of the above supernatant was placed in a test tube containing 0.5 ml of distilled water and 1.0 ml of diacetyl monoxine reagent (1% in acetic acid). Then 2.0 ml of oxidizing agents (10 mg Arsenic acid/ml in Conc. HCL) was added to the mixture. This mixture was incubated for 30 min in a water bath. After boiling and cooling at room temperature the optical density was measured at 475 nm.

Calculation of data

Activity of arginase enzyme was calculated as units/ml culture supernatant.

$$175.4 \mu\text{g Urea} = 100 \mu\text{g Arginase}$$

According to Sigma (Sigma Chemical Co., MO, USA) 50 units = 1 mg of solid Arginase. One unit converts 1.0 µmol of L-arginine to ornithine and urea/min at pH 9.5 at 37°C.

Assay for 5'-nucleotidase

The activity of 5'-nucleotidase was assayed in monocytes (5×10^5 cells/ml) cultured with medium alone or LPS by the method of Touster et al. 1970. 1.0 ml of M glycine buffer (pH 9.1) containing 0.005 M AMP as substrate and 0.01 M MgCl₂ was added to monocytes and incubated at 37°C for 30 min. Blank controls containing monocytes without substrate or only substrate without monocytes were also run and incubated in the similar manner. Reaction was stopped by adding 2.0 ml of 8% TCA and kept at room temperature for 10 min. The supernatant were removed in centrifuge tubes, which were centrifuged at 4000 rpm for 10 min. The supernatants were stored for estimation of inorganic phosphorus (Pi) by the method of Fiske and Subbarow (1925).

Estimation of inorganic phosphorus

5'-nucleotidase activity was measured as µg inorganic phosphate (Pi) released/ml cell lysate. For estimating Pi, 5 ml of distilled water of 5 N H₂SO₄, 1.0 ml of ammonium molybdate 2.5% and 0.5 ml of reducer (prepared with 1.2 g Na₂SO₄, 1.2 g Na₂SO₄ and 0.2 g 1,2,4-Amino Naphthol sulphonic acid, ground in pestle mortar) was added to the 0.5 ml of supernatant in duplicates mentioned above. To this, distilled water was added slowly and the volume was made up to 100 ml and stored in brown bottle at 4°C. The sample tubes were kept at room temperature for 30 min and the optical density was measured at 660 nm. Relative increase or decrease in optical density for inorganic phosphate (Pi) release showed respective increase or decrease in enzyme activity.

Calculation of data

5'-Nucleotidase activity was measured as, µg Pi released/ml cell lysate/ 5×10^5 cells.

Assay for lysozyme

Lysozyme assay was performed with culture supernatants collected monocytes by the method described by Barret and Heath (1977). A substrate suspension of the dried and killed *Micrococcus lysodeiticus* (0.1 mg/ml) was prepared in 0.1 M sodium phosphate buffer, pH 6.2 to give E₆₀₀ in the range of 0.330–0.340 at 37°C. Blank controls with 2.0 ml of buffer run to set the zero. To each tube containing 2.5 ml of substrate solution, 100 µl of culture supernatant was added. The decrease in optical density at 600 nm was measured after incubation of tube in water bath at 37°C for 1 h.

All the experiments were done in triplicates and results expressed as in units/ml culture supernatant. Lysozyme units were calculated as change in absorbance/min $\times$ 1000.

$$\text{Or} = \frac{\text{Change in absorbance}}{\text{Minute}} \times 1000$$

$$= \text{Units/ml culture supernatant.}$$

Statistical analysis

Power analysis was applied wherever necessary.

Results

Study population

The clinical and laboratory features of the 15 patients on admission are summarized in Table 1. All the cases had a history of fever, leucopenia, thrombocytopenia, low hemoglobin and hepatosplenomegaly (Table 1). Leukocytes counts ranged between 4000 and 7000 mm^3 . None of cases had lymphadenopathy. The parasites counts in the splenic aspirate ranged 2+ to 5+ (Chulay and Bryceson 1983).

Arginase activity in monocytes

Monocytes were from healthy controls and active VL patients. Monocytes and their supernatants were collected for estimation of arginase. Monocytes of active VL patients showed significantly low arginase activity as compared to healthy controls (Table 2). Increased arginase activity but not significant, was observed in monocytes from active VL patients on treatment with LPS ($\mu\text{g/ml}$) as compared to healthy controls.

5'-Nucleotidase activity in monocytes

Culture supernatants collected from monocytes isolated from active VL patients and healthy controls were estimated for 5'-nucleotidase activity. Monocytes from active VL patients showed significantly high 5'-nucleotidase activity as compared healthy controls (Table 2). Low but not significant 5'-nucleotidase activity was observed in active VL patients on treatment with LPS. 5'-nucleotidase activity of monocytes from healthy controls significantly decreased on treatment with LPS (10 $\mu\text{g/ml}$) as compared to monocytes of active VL patient.

Supernatant collected from cultured monocytes of active VL and healthy controls were assayed for lysozyme activity. Monocytes isolated from healthy controls showed significantly high lysozyme activity as compared to active VL patients (Table 2). Significantly low lysozyme activity

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

S. no.		Study entry	
		VL patients	Controls
1.	No. of patients	15	10
2.	Age (years)	23.3 $\pm$ 3.58	27.2 $\pm$ 3.4
3.	Duration of illness (months)	4.43 $\pm$ 0.38	–
4.	Weight (kg)	44.5 $\pm$ 5.58	59.0 $\pm$ 5.5
5.	Karnofsky score*	77.0 $\pm$ 1.68	100
6.	Spleen size (cm)	8.27 $\pm$ 1.42	–
7.	Splenic aspirates score#	2.45 $\pm$ 0.38	–
8.	WBC count ($\times 10^3/\text{mm}^3$)	3.68 $\pm$ 0.78	8.6 $\pm$ 1.2
9.	Hemoglobin (g/dl)	9.10 $\pm$ 0.58	14.1 $\pm$ 1.7
10.	Platelet count ($\times 10^3/\text{mm}^3$)	0.76 $\pm$ 0.23	184.0 $\pm$ 21.1

* Karnofsky performance scale

Able to carry on normal activity: 100 Normal, no complaints, no evidence of disease

no special care is needed

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 need, a amount of assistance is needed 70

Care for self, unable to carry on normal activity or to do active work

Splenic aspirates score

Grading of the parasites (Chuley and Bryceson 1983)

0	0	Parasites/1000 field
1+	>1–10	Parasites/100 field
2+	>1–10	Parasites/100 field
3+	>1–10	Parasites/10 field
4+	>1–10	Parasites/field
5+	>10–100	Parasites/field
6+	>100	Parasites/field

by monocytes of active VL patients was observed when treated with LPS (10 $\mu\text{g/ml}$) as compared to monocytes of controls treated with LPS (10 $\mu\text{g/ml}$).

Discussion

Intramacrophage infection by *L. donovani* results in potentially fatal visceral infection in humans and the elimination of *Leishmania* parasites by the macrophage is dependant upon the mounting of an effective cell mediated

Table 2 “In vitro” arginase, 5'-nucleotidase and lysozyme activity monocytes from active VL patients and healthy controls

Status	Arginase activity (U/ml)	5'-Nucleotidase activity (μmol Pi released/ml cell lysates/2 × 10 ⁵ cells)	Lysozyme activity (U/ml)
Acute VL patients (n = 15)			
Medium alone	5.43 ± 0.321*	34.40 ± 0.390**	0.200 ± 0.008***
LPS (10 μg/ml)	6.37 ± 0.34	30.03 ± 0.359	0.256 ± 0.014
Uninfected (n = 10)			
Medium Alone	7.65 ± 0.315	8.27 ± 0.145	0.285 ± 0.001
LPS (10 μg/ml)	6.17 ± 0.428	7.73 ± 0.218 [#]	0.582 ± 0.003

* Significantly decreased over uninfected controls $P < 0.001$

** Significantly increased over uninfected controls $P < 0.001$

*** Significantly decreased over uninfected controls $P < 0.01$

[#] Significantly decreased over active VL patients $P < 0.001$

immune response by the mammalian host (Locksley and Louis 1992).

The macrophage is the exclusive host cell of the intracellular protozoan parasite *Leishmania*, where it multiplies and survives despite the microbicidal environment of mononuclear phagocytes. It was of interest to study effect of *Leishmania donovani* infection on activation capacity of mononuclear phagocytic cells of kala-azar patients. The term activation is commonly used to describe heightened state of macrophage function that is associated with an increased capacity of these cells to kill intracellular and extra cellular targets (Pearson et al. 1983; Locksley and Louis 1992).

In an earlier study we investigated, reactive oxygen intermediate(s) (ROI), nitrite and interferon-gamma (IFN- γ) production by monocytes from active VL patients. We found that at different times during treatment in patients with visceral leishmaniasis (VL), hydrogen peroxide (H₂O₂), superoxide (O₂⁻) and IFN- γ production by cultured monocytes were significantly lower as compared with the healthy controls. In contrast, nitrite levels in the supernatants from monocytes cultures of VL patients were comparable to healthy controls and increased significantly during anti-leishmanial therapy. On day 20 of treatment, a significant increase in release of H₂O₂, O₂⁻ and IFN- γ was observed. However, at follow-up, 4 months after the end of treatment, the production of H₂O₂, O₂⁻, IFN- γ and nitrite had declined significantly. Thus, the impairment in hydrogen peroxide and superoxide production suggested that down-regulation of these mediators may be involved in the reduced killing of parasites by monocytes of active VL patients (Kumar et al. 2001). In a separate study low levels of NADH and NADPH-oxidase and myeloperoxidase activity was observed in monocytes isolated from acute VL patients supporting our observation of impairment of respiratory burst activity (Kumar et al. 2002). A recent

study shows that the increase in peroxide and superoxide production kills the parasite (Shukla et al. 2011).

Arginase has been considered as an effector product of activated macrophages. Arginase and NO synthases (NOS) are both LPS inducible enzymes in macrophages that act on a common substrate, arginine (Granger et al. 1990). Thereby, arginine can be metabolized in macrophages by NOS to nitric oxide and citrulline or by arginase to ornithine and urea.

Low levels of arginase activity were observed in monocytes from active VL patients as compared to healthy controls. However, LPS treated monocytes of VL patients showed slight increase but not significant, arginase activity as compared to individual patients monocytes obtained from VL patients. There are reports available dealing with the suppressive effect of macrophage derived arginase on the cellular proliferation; whether the target cells were lymphocytes, tumor cells, parasites or viruses (Schneider and Dy 1985). Hibbs et al. (1987) proposed a function for macrophage arginase resulting nitrogen oxidation reaction. Low levels of arginase production by monocytes may account for increased proliferation of *Leishmania* parasites in active VL patients.

5'-nucleotidase has been cited as a biochemical criteria of macrophages an altered morphological and biochemical state of activation and stimulation (Edelson and Cohn 1976a, b; Karnovsky and Lazdins, 1978; Pietrangeli et al. 1981). A decline in macrophage 5'-nucleotidase level is associated with activation by *Coryne bacterium parvum* and BCG (Karnovsky and Lazdins 1978; Bruley-Rosset et al. 1976) and stimulation by sodium caseinate and thioglycollate (Edelson and Cohn 1976a, b). 5'-Nucleotidase activity has been found to be inhibited by different activators like cytoplasmic protein antigen, endotoxin (Evans and Alexander 1976) LPS, bacterial extracts (Schorlemmer et al. 1977) and mycobacterial cell wall. Depression in

5'-nucleotidase activity has been generally referred to as a characteristic marker of activated macrophages (Karnofsky and Lazdins 1978). Our results showed high levels of 5'-nucleotidase in cells from active patients, thereby confirming impairment of macrophage activation as a result of parasitization by *Leishmania*. Whereas, normal levels of 5'-nucleotidase activity was observed in healthy endemic and non-endemic controls. Further monocytes from healthy controls on treatment with LPS showed an up-regulation in 5'-nucleotidase as shown by low levels of 5'-nucleotidase.

According to Werb and Cohn (1972) low levels of 5'-nucleotidase is a result of inactivation of the enzyme by other lysosomal enzymes during membrane internalization and phagosome-lysosome fusion during phagocytosis. It is reported that 5'-nucleotidase which is particularly rich in resident macrophage is lost progressively with activation. In the activated cells its rate of degradation is enhanced and its synthesis is unable to keep with these demands.

Lysozyme activity was also measured in supernatant collected from monocytes of VL patients and healthy controls. Low lysozyme activity was seen in supernatant of monocytes obtained from active patients. However, there was a dramatic effect of LPS on lysozyme activity in healthy controls monocytes versus non-stimulated cells from the same individuals. The effect was not observed with VL patients. Lysozyme or lysosomal enzymes are released into the endocytic or azurocytic vacuoles thereby serving the purpose of intracellular digestion of engulfed or segregated materials. These lysosomal enzymes may escape from the cell when lysozymes fuse with incomplete vacuoles during phagocytosis. This phenomenon has been well documented in polymorphonuclear leucocytes (PMN) as reported by Weismann et al. (1975).

It has been reported that in variety of clinical condition plasma and urinary lysozyme levels are elevated (Perrillie and Finel 1973). Jolles and Jolles (1967) have reported that lysosomal enzymes and lysozyme are involved in the defense functions of the body. This enzyme is particular interest in the study of phagocytic cells, as one of the main functions of these cells is to localize the microorganism in the body of host then degrade and eliminate them. While there are some facts known, most of the theories regarding the immunopathogenesis of kala-azar rely on inferences from human cutaneous leishmaniasis and clues obtained from murine models. No model mimics the various forms of human disease perfectly. Moreover, scant studies are available on the aspect of macrophages activation in human VL infections. This is the first study in our knowledge which has investigated arginase, 5'-nucleotidase and lysozyme activity in active VL patients.

We conclude that there is a down-regulation of macrophages activation (referred to by biochemical parameters of macrophage activation i.e. low arginase and lysozyme and

high 5'-nucleotidase activity) in the pathogenesis of VL infection. Further studies are warranted to elucidate the mechanisms especially the role of second messengers and signal transduction in the down-regulation of macrophage activation by *Leishmania* parasites.

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