American Journal of Immunology 1 (3): 96-100, 2005
ISSN 1553-6203

Towards Identifying Immunogenic Targets in Visceral Leishmaniasis: Role of 17kDa and 63kDa Phosphoproteins

[1]Shubhankar K Singh, [1]Sanjiva Bimal, [1]Diwakar S Dinesh, [1]Anil K Gupta, [1]Prabhat K Sinha, [2]Raajeeva Bimal and [1]Pradeep Das.
[1] Rajendra Memorial Research Institute of Medical Sciences (ICMR), Patna-800007, India.
[2] Biotechnology Lab., University Department of Biotechnology, BRA Bihar University, Muzaffarpur, India

Abstract: We observed predominant phosphorylation at Protein Kinase C (PKC-α) residue in seven immunoreactive proteins of virulent *Leishmania donovani* parasites. These phosphoproteins were tested for their ability to stimulate T-cells for secretion of Macrophage Migration Inhibition Factor (MIF) in comparision to crude soluble antigen in Visceral Leishmaniasis (VL) patients. Two of these phosphorylated *L. donovani* antigens led *Leishmania* to present these antigens to Th1 cells, which was obvious from an increased MIF response. These phosphorylated antigens i. e. 63kDa and 17kDa further led to reprogramme CD4 cells for enhanced IFN-γ production. The study suggests that PKC-phosphoryalation in *L. donovani* antigens can play a critical role in modulation of signal transduction pathway in T-cells to differentiate into Th1 effectors.

Key words: Protein Kinase C, Macrophage Migration Inhibition Factor, *Leishmania donovani,* Interferon Gamma, Visceral Leishmaniasis.

INTRODUCTION

The eukaryotes are reported to employ a signalling mechanism at tyrosine residue that controls a variety of intracellular events[1]. Virulent promastigote strain of *Leishmania* also undergo phosphorylation of tyrosine containing proteins and as reported, it is species specific[2]. Reports on tyrosine related proteins, protein kinase C (PKC), a serine/threonin containing protein are also available which suggest its role in innate macrophage functions and hence thereby, appears to be a key element in the control of infection by *L. donovani*[3]. The reports presented in this study describe the phosphorylated or dephosphorylated status of differentially expressed *L. donovani* antigen grown initially at 24°C and later subjected to 30°C and 37°C temp. shift in laboratory based experimental set-up. Such heat shock induced changes were further compared with the antigenic pattern shown by natural promastigotes present within *Phlebotomus argentipes*. The investigations were further carried out to assess the antigenic components for their immunological properties.

MATERIALS AND METHODS

Promastigotes pass through a rapid temperature shift from 22-24°C to 37°C while entering in mammalian host through *P. argentipes* the proven vector of Indian Visceral Leishmaniasis. As such, late log phase *L. donovani* culture (3-4 days) propagated in M-199 medium with 10% FCS was incubated at 24° C and 37° C for 4h. This was done to experimentally mimic those conditions that usually are governed in vivo during conversion of promastigotes into amastigotes. To study antigenic pattern of *Leishmania* during natural infection, *P. argentipes* (10/pool in 50µl of distilled water) were collected from three adjacent endemic areas from Bihar province in India. A 12.5% SDS-PAGE[4] was later applied for proteins of 50µg lysates/lane and antigenic pattern of promastigotes at 24, 30 and 37°C and those present within *P. argentipes* was compared. Later, antigens blotted from different experimental stages were probed with anti-PKC-α antibodies (mouse IgG 2b; clone 3, BD Biosciences, USA) in TBS-tween (1:1000; 0.25µg/ml final concentration) that followed incubation with IgG-HRP (1:2000 dilution in wash buffer containing 1%BSA) for 1h at room temperature, which were stained with 0.3% diaminobenzidine chemiluminiscent working solution for 1-5 min. (0.3% diaminobenzidine). Colour development was monitored and the reactive bands were calculated for their respective molecular weight size. These antigens with a critical role in signalling, were further tested for their effect on humoral immune response to *L. donovani* for which NCP blotted antigenic fractions were probed with pooled sera in immunoblot[5]. These sera were obtained from 10 VL subjects in whom infection was confirmed microscopically by demonstration of amastigotes in

Corresponding Author: Ishag Adam, Faculty of Medicine, University of Khartoum, Sudan, Tel: +249 912770277
Tel: +249 183 224762, Fax: +249 183 2247 99

spleen biopsy and in whom *Ld* grade ranged from 1+ to 5+. These subjects were also confirmed as VL through PCR and DAT. For the study of T-cell response SDS-PAGE slots of selected antigens were isolated and subjected to second SDS-PAGE gel and subsequently transferred on NCP. The polypeptides on blotted NCP was stained with amido-black (0.5% amido-black, Hi-media, India with 25% methanol and 7% acetic acid in distilled water), isolated into PBS and stored at –70°C for further studies. To study the association of T-cells in response to these antigens, $5x10^6$/ml peripheral blood mononuclear cells (PBMNC) were pulsed with *L. donovani* promastigotes in responder (PBMNC) to stimulator (*L. donovani* parasite) ratio of 100:1 for two hours at 37°C and later cultured in 96 well round bottomed plates in presence of selected polypeptides (1μg/100μl culture). Control cultures were set up in medium alone or medium containing soluble *Leishmania donovani* 20μg/ml for 48h. All cultures were maintained at 37°C in a water saturated air atmosphere containing 5% CO2. Macrophage migration inhibition factor (MIF) was measured as an index of cell mediated immunity[6]. Two of these protein fragments (17kDa and 63kDa) which showed an augmented T-cell response for MIF were further tested for their abilities to stimulate intracellular IFN-γ production by $CD4^+$ subpopulation on FACS-Calibur using cell quest software. Briefly, the cells stimulated with 17kDa and 63kDa protein fractions in presence or absence of recombinant IL4 for 18h at water saturated atmosphere in 5%CO_2 incubator. Cultured cells, pre-treated with Brifeldin-A for 6h before harvesting, were fixed, permeabilised and stained with anti CD4 PE and anti-IFN-γ-FITC as previously described[7].

RESULTS

The results show appearance of 3 proteins upon heat stress from 24°C to 30°C, which remains stable at 37°C. The polypeptides identified ranged in the molecular weight of 6.5 to 87 kDa as determined on the basis of mobility of stander marker. Most of the polypeptides appeared at 24°C were also visible in the SDS-PAGE pattern of *P. argentipes* while few proteins of sand-fly (57kDa, 40kDa, 15kDa etc) did not match Leishmanial pattern under any condition of the heat stress and thus probably represented sand-fly specificity of these proteins (figure-1). Predominant phosphorylation in 15 polypeptides was observed at PKC-α residue (24°C) but 4 of these proteins did not phosphorylate when temperature was shifted to 30°C (Table-1). A new polypeptide of 21kDa molecular size showed significant phosphorylation at 30°C but this antigen suddenly got dephosphorylated at 37°C. Altogether 7 important proteins with significant impact on phosphorylation state was identified which were of molecular size of 63, 39, 32, 26, 24, 19 & 17 kDa. To assess the immunogenic properties of these critical *Leishmania* antigens, humoral immune response for elicitation of anti-Leishmania donovani antibodies in response to these antigens was examined by Western blot. These antigens induced strong antibody response but many antigens which earlier showed poor phosphorylation at PKCα residue also triggered antibody response in sera from VL patients.

In comparison, all phosphoproteins elicited antibody response but this type of response was not seen with majority of antigens where no phosphorylation was observed at PKCα residue.

We later examined cytokine response (MIF& IFNγ) to whole or fractionated phosphoprotein *L. donovani* antigens. Increased humoral response in *L. donovani* phospho-proteins was later observed associated with raised T-cell function for MIF response (Table-2). MIF release up to 46.09% was observed after priming of PBMNC with 17kDa (10μg/ml) *L. donovani* polypeptide. This up regulated pattern of MIF expression was also noticed with 63kDa (44.12%), 24kDa (35%), 26kDa (33%) and 19kDa (27.5%). The above response however, was not observed when PBMNC were primed with crude soluble antigen where T-cells secreted insignificant MIF of 16.78%. To further investigate role of these phosphoproteins, IFNγ production by CD4+T-cells induced by them was determined on FACS-Calibur after short-term stimulation.[7] Eighteen hours culture with these Leishmania derived antigens and CSA were analyzed using dual staining and flow cytometry. Analyzing lymphocytes in R1 gate on FSC Vs SSC plot, it was observed that CSA induced a high IFN-γ producing CD4 cells (2.8%) compared to medium (0.8%). When these results obtained with CSA were compared with *Leishmania* derived antigens, there was an increase in frequency of IFN-γ producing cells, which was 5.28% with 63kDa and 5.11% with 17kDa (Figure-2). The response of 17kDa was slightly better as it induced higher frequency of IFN-γ producing cells compared to 63kDa when cultured in presence of recombinant IL4.

DISCUSSION

It is well known that PKC-α, a calcium dependent PKC isoform regulates innate macrophage function, which is involved in the control of infection by *L. donovani*[3]. Recently it has been shown that VL infection is associated with selective impairment of PKC isoform caused by *Leishmania donovani* parasite which results in dysfunction in Ca^{2+} signalling[8,9]. Many reports also suggest the involvement of PKC mediated phosphorylation in CD4 cells, which results in proliferation and IL2 production and its receptor

expression[10,11]. Therefore we speculated that there might be some activation in PKC dependent signalling in certain antigens of *L. donovani,* many of which, we thought can be with pro-host immuno-modulatory action. To characterize the role of *Leishmania* antigens, which may be used to produce a protective immune response, we examined the phosphorylation of different antigens at PKC-α residue and later examined its impact on MIF and IFN-γ response of T-cells. The PKC-α phosphorylation was observed up regulated in 7 *Leishmania donovani* antigens and the increased phosphorylation possibly led *Leishmania* to present these antigens to Th1 cells, which was obvious from increased MIF response. MIF secretion by T-cells was recently shown by our group to act as a prognostic marker of VL infection[12]. The results highlights the importance of two critical antigens i. e. 63kDa and 17kDa, which were shown better, shaped in reprogramming of CD4 cells to produce IFN-γ in VL patients. The trends obtained further suggest that 17kDa antigen can be a promising immuno-therapeutical target, as there seems to be some mechanism triggered by this antigen in detecting IL4 as a result of which this cytokine was interrupted to prevent the production of IFN-γ up to some extent. It is now well known that outcome of leishmanial infection relies on induction of type-1 response of CD4 cells, which produce IFN-γ[13,14]. The study presented here reflects that triggering of PKC in *L. donovani* is important which can enforce re-stimulation in T-cells for Th1 sub-set expansion. The inability of most vaccine formulations to prevent the infection has been the feature observed for most experimental vaccines against leishmaniasis so far. Hence evidence for a role of 17kDa and 63kDa in immunity to *Leishmania donovani* infection shown in this study is worth pursuing. The information collected on immunogenic properties of these antigens will be useful in studies based on immuno-modulatory therapy and vaccine development.

ACKNOWLEDGEMENTS

The technical assistance rendered by Mr. Arbind Prasad during the course of this work is highly acknowledged.

Table-1: Characteristics of stable protein fractions under western blot analysis:

	Proteins fractions recognized by sera from VL patients	Proteins fractions not recognized by sera from VL patients
Proteins fractions recognized by PKC-α residue	63, 39, 32, 26, 24, 19 & 17	-
Proteins fractions not recognized by PKC-α residue	87, 71, 49 & 29kDa	77, 65, 43.5, 28, 20.5, 20, 18.5, 18, 15 kDa

*The 7 protein fractions showing strong humoral response were reactive to PKC-α residue, however, 4 immuno-reactive protein fractions did expressed their reactivity to PKC-α residue. 9 protein fractions showed their reactivity neither with patient sera nor with anti PKC-α antibodies.

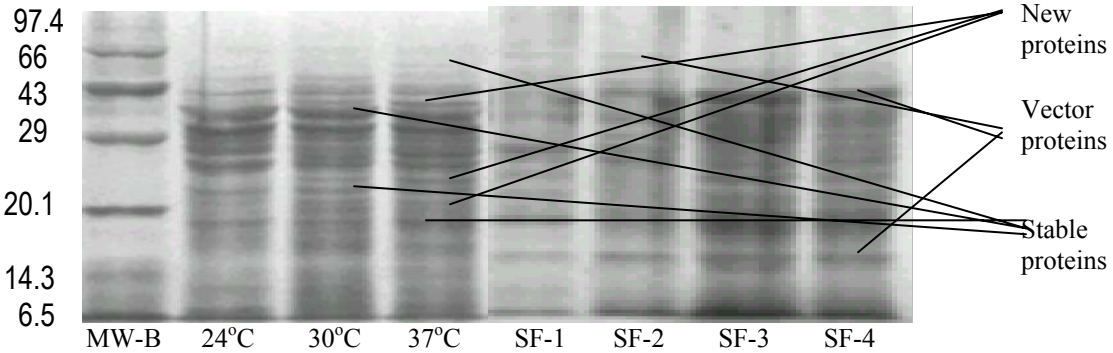

Fig. 1: SDS-PAGE pattern of *L. donovani* isolates cultured at 24°C , 30°C and 37°C in comparison to SDS-PAGE pattern of *P. argentipus* from endemic area

*SDS-PAGE pattern of crude *L. donovani* promastigote cultured at 24°C (L-2) 30°C (L-3) 37°C (L-4), compared to SDS-PAGE pattern of *P. argentipes* lyssate (10/pool dissolved in 50µl distilled water) collected from VL endemic areas (lane 5-8, labelled as SF1, SF2, SF3, SF4). There are expression of few newly appeared proteins on lane 3 and 4. Majority of proteins are stable on heat shock and also visible in lane 5-8. However some of the proteins of lane 5-8 did not matched with Leishmania pattern of any heat stress condition and thus probably represented sand-fly proteins under this range.

Table-2: MIF response by T-cells from VL and healthy subjects after priming with different immunogenic protein fractions from *L. donovani*

Category	Crude soluble antigen of *L. donovani*	Fractionated proteins							PHA
		63 kDa	39 kDa	32 kDa	26 kDa	24 kDa	19 kDa	17 kDa	
Visceral Leishmaniasis (n=5)	16.78 ±5.17	44.12 ±7.44	12 ±1.14	14 ±4.24	33 ±2.82	35 ±5.65	27.5 ±4.94	46.09 ±6.05	36.55 ±6.19
Healthy (n=5)	12.12 ±2.61	19.22 ±4.56	9.5 ±3.53	10.5 ±2.12	18 ±2.82	14 ±5.65	9.5 ±3.53	18.9 ±1.27	36.51 ±4.1

Different polypeptide fractions showed differential impact on secretion of macrophage migration inhibition factor by T-cells in vitro.

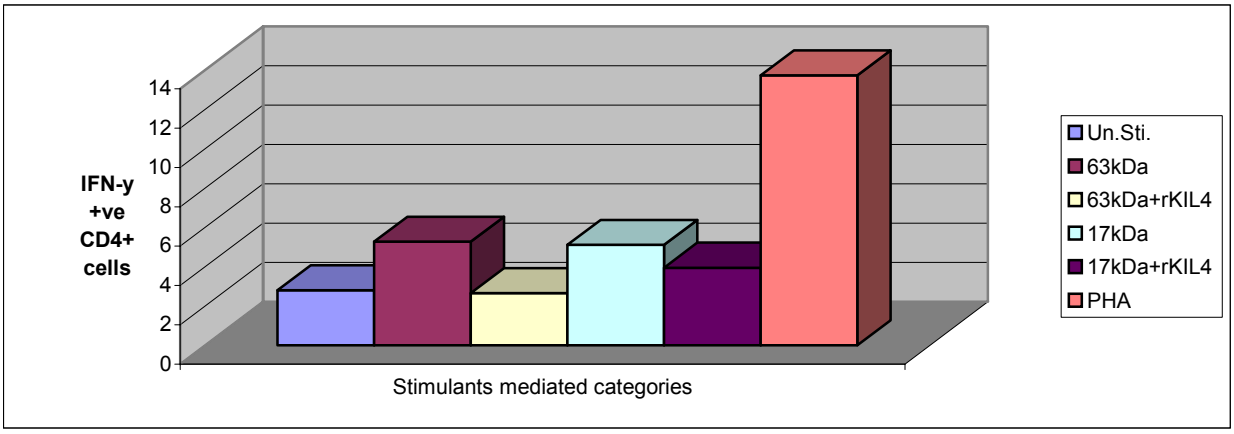

Fig. 2: Frequency of IFN-γ producing CD4+T-cells after stimulation with 63kDa or 17kDa in presence or absence of recombinant IL4.

*Increased frequencies of detectable IFN-γ producing CD4 cells after stimulation with 17kDa and 63kDa *Leishmania* derived antigens in presence or absence of recombinant IL4 and compared to crude soluble *Leishmania* antigen preparation and PHA. The response of 17kDa protein fragment was slightly better in presence of recombinant IL4.

REFERENCES

1. 1. Krebs, E.G., 1985. Phosphorylation of proteins: a major mechanism for biochemical regulation. Biochem oc Trans 13: 813-820.
2. Salotra, P., R. Ralhan and G. Sreenivas, 2000. Heat-stress induced modulation of protein phosphorylation in virulent promastigotes of *Leishmania donovani*. *The International Journal of Biochemistry & Cell Biology*.32.3, 309-316.
3. St. Denis, A., V. Caouras, F. Gervais and A. Descoteaux, 1999. Role of Protein Kinase C-α in the control of infection by intracellular pathogens in macrophages. *J. Immunol.* 163**:** 5505-5511.
4. Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
5. Towbin, H., T. Staehlin and J. Gordon, 1979. Electrophoretic transfer of polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the USA* 76: 4350-4354.
6. Clausen, J.E., 1971. Tuberculin induced migration inhibition of human peripheral leucocytes in agarose medium. *Acta Allergol* 26: 56-80.
7. Prussin, C. and D. Metcalft, 1995. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J Immunol Meth* 188: 117-128.
8. Moore, K.J., S. Labrecque and G. Matlashewski, 1993. Alteration of *Leishmani donobani* infection level by selective impairment of macrophage signal transduction. *J Immunol* 150: 4457-4465.
9. Bhattacharya S., S. Ghose, P.L. Jhonson, S.K. Bhattacharya and S. Majumdar, 2001. Immunomodulatory role of IL-10 in Visceral Leishmaniasis: Defective activation of Protein Kinase C- mediated signal transduction events. *Infect Immun* 62: 1058-1063.

10. Cantrell, D.A., A.A. Davies, M. Londei, M. Feldman and M.J. Crumpton, 1987. Association of phosphoregulation of T_3 antigen with immune activation of T-Lymphocytes. *Nature* 325: 540.
11. Droge, W., 1986. Protein Kinase C in T-cell regulation. *Immunol Today* 11: 341.
12. Bimal, S., S.K. Singh, V.N.R. Das, P. K. Sinha, A.K. Gupta, S.K. Bhattacharya and P. Das, 2005. *Leishmania donovani*: effect of therapy on expression of CD2 antigen and secretion of macrophage migration inhibition factor by T-cells in patients with visceral leishmaniasis. *Experimental Parasitology*. 111: 130-132.
13. Dasgupta, B., R.K. Chaudhary, S. Ganguli, P.K. Sinha, S. Vimal, P. Das and S. Roy, 2003. Anti-Leishmanial drugs cause up-regulation of interferon-gamma receptor 1, not only in the monocytes of Visceral Leishmaniasis cases but also in cultured THP1 cells. *Annals Trop Med Parasitol* 97.3: 245-257.
14. Thakur, C.P., D.K. Mitra and S. Narayan, 2003. Skewing of cytokine profiles towards T-helper cell type 2 response in Visceral Leishmaniasis patients unresponsive to sodium antimony glucinate. *Trans R Soc Trop Med Hyg* 86: 245-248.