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Attachment of *Leishmania donovani* virulent strains –Murine Peritoneal Macrophage interaction induces PKC mediated oxidative events

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Abstract
Leishmania donovani parasite proliferates within the host macrophage. Knowledge about the regulation of the oxidative metabolism in the host cells on virulent and avirulent strains of *L. donovani* and oxidative mechanisms after its attachment on the macrophage surface are still not clear. *In vitro* replication revealed that the degree of virulence is highest in GE-1 >AG-83 >UR-6 strains. It was observed that on parasite attachment, augmentation of O_2 consumption, O^{2-} production and NO generation occurred and these oxidative events diminished and differ significantly with intracellular replication of the parasites. Compared to UR-6, Protein Kinase C (PKC) activator-induced oxidative burst activity and bacterial LPS and IFN-γ induced NO production were markedly inhibited in peritoneal macrophages infected with GE-1 and AG-83 strains. Moreover, PKC inhibitor attenuates O^{2-} production and NO generation. NO generation was also inhibited by Protein tyrosine kinase (PTK) inhibitors, indicating the role of PKC and PTK in parasite mediated NO-signaling pathway. However, PKC activity in cell membrane fraction increased considerably and had a synergistic effect with PKC activator following attachment with virulent strains compared to infected conditions. Thus, impairment of PKC-dependent oxidative events by virulent strains of *Leishmania donovani* may attenuate peritoneal macrophage activation and contribute to persistence of infection.

Key words: *Leishmania donovani*, Macrophage, Attachment, Oxidative events, PKC, PTK

Introduction
The causative agent of visceral leishmaniasis or kala-azar, *Leishmania donovani*, is an obligate intramacrophage parasite that survives within the phagolysosomes. Although, the pathogen possess varied mechanisms for their intracellular compartmentalization, the host defense rely chiefly on the macrophage derived effectors molecules as reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), responsible for killing of these pathogens.
In regulation of a variety of cell function, PKC plays a major role. These kinases in cells are translocated and activated in response to hormones or phorbol esters. A well known phorbol ester, PMA, depending on varied incubation period, has been shown to activate PKC [1] from cells. The functional end points of this signal transduction cascade in phagocytic cells are superoxide generation [2]. Infection of macrophages with *Leishmania donovani* downregulates PKC dependent events in macrophages as PMA-stimulated oxygen consumption [3], NADPH-oxidase mediated superoxide generation [4] and nitrogen dependent mechanism [5]-[7]. It is still not clearly understood what happens to the oxidative events when the parasite just attaches onto the surface of macrophages before internalization. The present works aims to address degree of virulency in three different strains of *L.donovani* from in vitro replication, oxidative metabolism in the host cells on virulent and avirulent strains of *L. donovani* and oxidative mechanisms after the attachment with virulent strains of *L. donovani* on the peritoneal macrophage surface induced PKC mediated oxidative events.

2. Research Method
2.1 Media and Chemicals: RPMA 1640 with L-Glutamine, M199, heat inactivated fetal bovine serum, Cytochalasin D, Phorbol -12-myristate-13-acetate (PMA), Staurosporine, Tyrphostin AG126, crude IFN-γ, Superoxide dismutase, Lipopolysaccharide (LPS), ferricytochrome C and sulphanilamide were purchased from Sigma Chemical Co.(USA).

2.2 Parasites: *Leishmania donovani* virulent strains GE-1(MHOM/IN/89/GE-1) and AG-83 (MHOM/IN1983/AG-83) were maintained in female BALB/c mice. *Leishmania donovani* avirulent strain UR6 were maintained and grown in modified Ray's medium.

2.3 Peritoneal macrophages: Murine Macrophages for infection studies were isolated by peritoneal lavage from female BALB/c mice as described [8].

2.4 Parasite attachment and infection of macrophages: Macrophages (10^6/ml) were treated with the drug Cytochalasin D (1.5µg/ml) for 1hr at 37°C before infection and continued to the end of incubation. Macrophages were then challenged with parasites (for 2hr at 37°C at 10:1 parasite -to-cell ratio). Infection with parasites was done for various time periods at 37°C. Non-interacted parasites were removed by three washes with warm PBS. In drug treated macrophages, parasites were found to be attached on macrophage surface, whereas in drug untreated macrophages most of the parasites were found to be internalized. Infection levels were determined by microscopic examination of Giemsa-stained slides.

2.5 Treatment of macrophages with various kinase inhibitors: Macrophage culture medium was removed from the cells on slides and replaced with fresh medium containing PKC or PTK inhibitors at various concentrations for 30mins at 37°C.

2.6 Oxygen consumption by macrophages: After adherence and treatment with parasites, macrophages were scraped by disposable cells scraper and suspended in Krebs-Henseleit buffer. PMA-induced O_2 consumption was measured polar graphically with Clark-type electrodes as described [9].

2.7 Measurement of superoxide and nitrite in presence of Protein kinase C or Protein tyrosine kinase inhibitors: O^{2-} was measured by the superoxide dismutase inhibitable reduction of ferricytochrome C as described [10]. O^{2-} release was determined spectrophotometrically at 550nm.The production of NO by macrophages was estimated by measuring the accumulation of nitrite, by the Greiss reagent. In brief, both normal and infected macrophages were treated with T-cell supernatant (con-A treated) as a source of IFN-γ for 24hrs and then with LPS, 5µg/ml for another 24hr. Cell supernatants were then mixed with equal volume of Greiss reagent (0.5% sulphanilamide and 0.05%-N-1-napthylenediamine hydrochloride in 50% phosphoric acid) and nitrite was measured spectrophotometrically at 550nm.

2.8 Preparation of Membrane fractions for PKC Assay: Thioglycollate elicited mice peritoneal macrophages were collected after incubation for 37°C then washed with PBS. Macrophages (10^6/ml) were stimulated with or without PMA (100ng/ml), treated with or without the drug Cytochalasin D (1.5µg/ml for 1hr at 37°C) prior to infection with parasites (for 2hr at 37°C at 10:1, parasite -to-cell ratio) and continued to the end of incubation. Non-interacted parasites were removed by three washes with warm PBS. Then the cells were sonicated in buffer containing 0.25mM sucrose, 5mM EGTA, 20mM HEPES, Leu–pep 50µg/ml, PMSF 0.2mM and 2mM β-mercaptoethanol. Sonicate was centrifuged at 100,000x g for 30min. The pellet was resuspended in buffer supplemented with 1% (v/v) Triton-X 100 and centrifuged at 100,000xg for 30min. The detergent-treated supernatant fraction, i.e., the membrane fraction was assayed for PKC activity as described [11] using Histone III as protein substrate. Briefly, activity of PKC was measured as the incorporation of ^{32}P from ^{32}P ATP into histone III at 37°C in the presence of 10mM $MgCl_2$, 20mM Tris-HCl, pH7.5, 50µM ATP (containing approx. $6x10^5$ cpm ^{32}P), 40µg histone, 0.4 mM EGTA, 0.2mM PMSF,10mM β-mercaptoethanol, 0.01% Triton X-100, 1mM $CaCl_2$, 20µg/ml PS and 2µg/ml Diolein. Samples were assayed in duplicate in both presence and absence of Ca^{2+}, PS and Diolein. PKC activity was determined by subtracting from the maximal apparent activity the amount of ^{32}P incorporated into histone in the absence of essential cofactors and expressed as percentage (%) ^{32}P incorporation/mg of protein [4], [11].

3. Results and Analysis

3.1 Replication of Intracellular *Leishmania donovani* parasites [Fig.1, peritoneal macrophage infected with GE-1 strain]
Mouse peritoneal macrophage were challenged with *L.donovani* strains GE-1, AG-83 and UR6 for various time periods as indicated in Fig. 2. The no. of parasites/100 macrophages increased by 3 to 5 fold for GE-1 and 2 to 3 fold for AG-83 at 24hrs and 48hrs and began to rise again to give an almost 7 fold increase for GE-1 and 5 fold for AG-83 by 72hrs [Fig. 2]. In contrast, for UR6 strain the parasites gradually dropped and by 72hrs the parasite count dropped ~50%. Thus, replication study showed that GE-1 was the most virulent strains tested.

3.2 Oxidative burst activity of *Leishmania donovani* attached and infected peritoneal macrophages and the role of PKC [Fig.3A & 3B]
Following phagocytosis, mononuclear phagocytes undergo respiratory burst, thus the superoxide generation was measured for peritoneal macrophages infected with virulent or avirulent *L. donovani* promastigotes. Superoxide (O^{2-}) production was significantly attenuated in macrophages infected with GE-1 and AG-83 strains compared to strain UR6 (O^{2-} with GE-1 and AG-83 were 38.33± 2.23 and 48±2.13 nmoles/mg of protein compared to 109.33±7.9nmoles/mg of protein for UR-6 and 81±3.41 nmoles/mg of protein for normal) [Fig. $3B_B$]. In contrast direct activation of PKC with PMA, also a known stimulus for O^{2-} production, increased O^{2-} production by control cells [Fig. $3B_A$]. However, in presence of Cytochalasin D, when parasites (GE-1 and AG-83) were allowed to attach onto macrophage surface, O^{2-} generations were significantly increased for both the virulent strains, whereas O^{2}- production was markedly diminished (~46% inhibition) in presence of PKC inhibitor, Staurosporine, but not in presence of protein tyrosine kinase inhibitor, Tyrphostin AG126 [Fig. $3B_A$], indicating the role of PKC in the process. The result showed that although parasite attachment induced O^{2-} generation, these generations were considerably inhibited during parasite multiplication and maximally inhibited for most virulent strain GE-1.

3.3 *Leishmania donovani* attachment does not regulate oxygen consumption [Fig.4]:
Inhibition of O^{2-} production in infected macrophages could be due to activation of oxidative burst or due to scavenging of reactive oxygen intermediates. To examine this, O_2 consumption was measured for normal, infected and parasite-attached peritoneal macrophages after PMA treatment. Compared to normal macrophages, O_2 consumption by macrophages infected with GE-1 and AG-83 was reduced by ~40-50% and for UR6 there was 4-5% stimulation in O_2 consumption. In contrast, attachment of different strains of *L. donovani* parasites to macrophages in presence of Cytochalasin-D [Fig.1B], induced the oxygen consumption substantially (6.69±0.10nmol/10^6 cells for GE-1 attachment compared to 2.93 ±0.091 for GE-1 infection and for AG-83 this was 6.22±0.1 during attachment versus 3.54±0.16 at infection and for UR-6 it was 7.75±0.15 during attachment versus 5.85±0.04 at infection). The results indicated *L. donovani* attachment to macrophages by all three strains induced almost similar level of O_2 consumption, there was significant reduction in consumption associated with the internalization and multiplication of the parasites and the reduction in consumption was inversely proportional to the virulence of the parasites.

3.4 NO generation during attachment and after infection [Table 1]:
To understand the role of NO in the multiplication of the intracellular parasites, activated peritoneal macrophages (crude IFN-γ primed and LPS- treated) with infected peritoneal macrophages was compared. The result showed that intracellular GE-1 parasites at 48hrs after infection inhibited the synthesis of NO (29.66±2.66µmoles/10^6 cells with GE-1 compared to 80.33± 4.31µmoles/10^6 for control activated macrophage and 70.66±3.10µmoles/10^6cells for UR-6). Thus, for the virulent strain GE-1 infection, the NO synthesis was inhibited by ~65%, whereas UR6 produced almost

similar amount of NO like control macrophages. But, significant production was observed during attachment of GE-1 to macrophage (110.66±3.10µmoles/10^6 cells). From the study, it was observed both PKC and PTK plays a significant role in NO production, as the effects of PKC inhibitor, Staurosporine inhibited NO release by ~40-42% and PTK inhibitor, Genestein and Tyrphostin inhibited ~64%, NO release. Thus, *L. donovani*-attachment induced the release of NO both in PKC and PTK –dependent manner. And probably after infection with virulent strains of *L. donovani* parasites, the activity of PKC and PTK to inhibit the production of NO was downregulated.

3.5 Effect of PKC activator (PMA) in peritoneal macrophage membrane protein during attachment and after infection: It was further examined the membrane fractions to assess the effects of parasite attachment and post infection on PKC activation. PMA, an agonist of PKC, known to activates or depletes PKC in different cell systems [12]. The result of PKC activity on parasite attachment and infection is shown in [Table-II]. Compared to normal peritoneal macrophage, PMA activated macrophage yielded more than 11fold increase in membrane associated PKC activity (2.43±0.13 versus 0.21± 0.05). Further, 4 times higher membrane associated PKC activity in parasite attached condition compared to infection with *L. donovani*, for both the virulent strains was observed. Moreover, a synergistic effect of membrane associated PKC activity was noted which was higher with PMA activated attached parasites compared to attached parasites only (for both the virulent strains, GE-1 and AG-83). Interestingly, for both the virulent strains of *L. donovani* under attached condition reduction of PKC activity was observed compared to peritoneal macrophage activated with PKC agonist, PMA.

Receptor interactions are important determinant of the attachment, entry, infectivity and survival of *Leishmania donovani* parasites in the vertebrate hosts [13]-[15]. The parasite attachment followed by entry involves sequential and circumferential interaction of receptors on the phagocyte with ligands on the surface of the parasite [16]. This process leads to the polymerization of F-actin at the site of ingestion and the internalization of the parasite [16]. *Leishmania* parasite resides and proliferates in phagolysosome of the host [17]. It is a known fact that the ability to express inducible activation-association functions that are normally involved in the elimination of microbes in *Leishmania*-infected peritoneal macrophages are rather impaired [18], [19], [20], [21]. During interaction of macrophage with *Leishmania* various signaling molecules are activated as PTK, PKC etc. [9], [20], [22], [23]. The phosphorylation of various proteins by PKC, in phagocytes of macrophages activates NADPH oxidase [24]. PKC mediated signal transduction cascade leads to the generation of superoxide (O^{2-}) in phagocytic cells as the functional end points of the mechanism [2]. PKC translocations effects on the phagocytic process [20], possibly by influencing events at the cellular cytoskeleton involved in parasite uptake and membrane trafficking [25]. In case of *L. donovani* infection, PKC the key enzyme for the generation of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) is impaired [9], [26], [27]. However, not much work has been reported in details during *Leishmania*-macrophage interaction, though significant work has been done after internalization [9], [28]. In the present study, before examining the oxidative events of macrophages affected with *L.donovani*, *in vitro* replication pattern was compared which depicted that infected macrophages got a 7-fold increase in parasite burden for GE-1 compared to 5-fold increase for AG-83. Microscopic examination of Giemsa stained infected macrophages suggested that after infection of 72 hours when multiplication of GE-1 and AG-83 parasites were induced but in UR6 parasites/100 macrophages dropped indicating that they were unable to multiply inside macrophages. Infection of macrophages with *L. donovani* attenuates several host functions including PMA-stimulated oxygen consumption [3], PKC mediated protein phosphorylation [29], stimulus response coupling through PKC [9]and Ca^{+2} homeostasis [30]. However during the early stage of recognition leading to attachment of parasites to macrophage, a significant stimulation of microbicidal parameters as induction in cellular activation through superoxide and nitric oxide generation in macrophages with virulent strains of *L.donovani* promastigotes was observed. Cytochalasin D, an inhibitor of actin polymerization, blocking

phagocytosis and having effects on intracellular trafficking as well, prior to infection with *L. donovani* virulent strains inhibits uptake but allow the attachment of parasites [31]. A number of bacteria are known to induce signal transduction based cytoskeletal rearrangement during attachment and entry [32], [33]. The study showed, during attachment of the virulent strain of *L. donovani* with mouse peritoneal macrophage, augmentation of O^{2-} production was considerably attenuated with the presence of Staurosporine but not with Tyrphostin AG 126. Hence, suggesting the role of PKC during attachment of virulent parasites with macrophages. However, after infection of macrophages with GE-1, significant downregulation in O^{2-} production was observed as compared to UR6.

It was earlier observed that most effective means of inducing activation in macrophages is through combined stimulation with a triggering agent like IFN-γ and bacterial LPS [34]- [36] and that a part of signal transduction apparatus involved in LPS-induced inducible nitric oxide synthase gene activation might be PKC [37].

This correlated with the present observation that IFN-γ activated LPS dependent stimulus triggered higher NO synthesis by *L. donovani* attachment in mouse peritoneal macrophage. In the study, at 48hrs of post infection, with GE-1 and AG-83, NO production was significantly inhibited compared to UR6 which was almost like normal macrophages. Downregulation of NO production by GE-1 and AG-83 strains might be due to inhibition of NO synthase signaling pathways by glycoconjugates of intracellular amastigotes form of the parasites [38], [39]. In contrast NO production under attachment condition (with Cytochalasin-D) was augmented compared to LPS+ crude IFN-γ primed macrophages and was inhibited in presence of Staurosporine and Genestein and Tyrphostin AG126 in parasite-mediated infection. But, the study observed a relatively higher PKC activity along with PKC induced transportation to the membrane during PMA induced attachment of virulent strains of *L. donovani* to mouse peritoneal macrophage surface compared to peritoneal macrophage attachment to the virulent strains (might be a synergistic effect of PKC and attachment of virulent strain of *L. donovani* to mouse peritoneal macrophage surface). It was also evident that much reduced PKC activation to the membrane during infected condition was recorded compared to macrophage attachment with *L.donovani* virulent strains. This correlated with earlier observation [4] where it was suggested this might be due to defective kinase activation in internalized condition. It was earlier observed that by attachment of the enzyme to membrane phospholipid in the presence of Ca2+ by the activation of PKC is typically accompanied by translocation of the enzyme to the membrane fraction **[**40]

Thus, though Staurosporine have the effect on other protein kinases and during attachment of parasites to macrophages, receptor-ligand pairing host and parasite surface might induce both PKC and PTK mediated NO signaling pathways as shown in [41], [42] but from translocation studies suggestion can be made that PKC mediated induction triggered production of superoxide and NO. Thus, increase superoxide and NO by the induction of *L. donovani* attachment might be due to the external stimuli induced elevated PKC activity often associated with elevated calcium and diacylglycerol levels [43].

Our work provided evidence that *L. donovani* virulent strains attachment signal differs once the parasite were internalized and also attachment of *L. donovani* virulent strains induced PKC mediated oxidative events.

Conclusion

These findings showed, that *L. donovani* virulent strains of parasites like any other microorganism when attached to the phagocytes induced PKC-mediated cellular events, however, after infection, only virulent strains significantly downregulated the oxidative events indicating attachment signaling was different from that of infection. The study showed that impairment of PKC-dependent oxidative events by virulent strains of *Leishmania donovani* may attenuate macrophage activation and contribute to persistence of infection. The study also indicated that attenuation of *L. donovani* induced PKC mediated oxidative events with virulent strains, helped in parasite survival and multiplication contributing to chronic infection.

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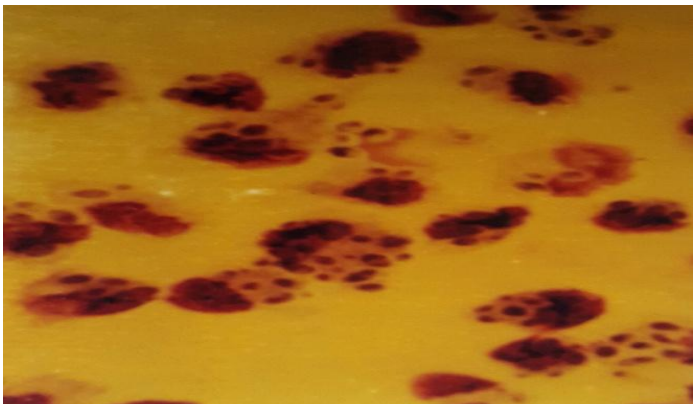

Fig.1A Mouse Peritoneal Macrophages infected with *L.donovani* parasites

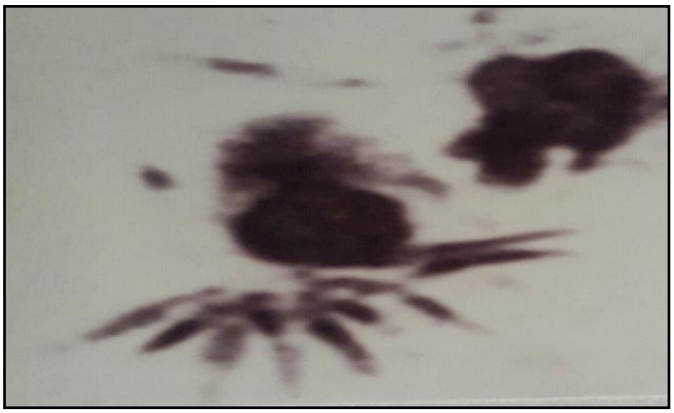

Fig. 1B Peritoneal Macrophages in attached condition with the treatment of Cytochalasin-D

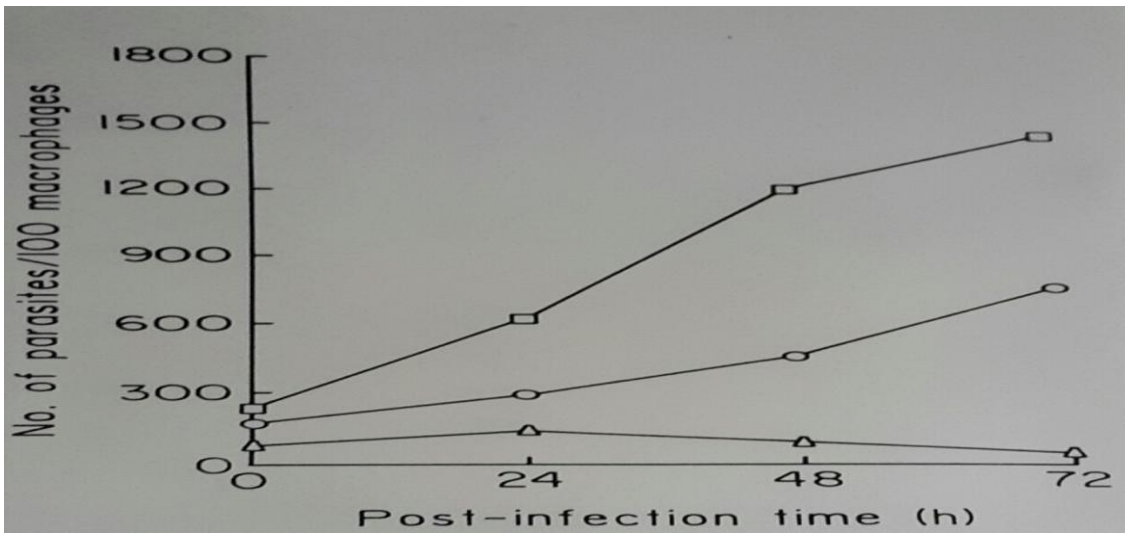

Fig.2 Intracellular replication of various strains of *L.donovani* promastigotes
Mouse peritoneal macrophages were exposed to pathogen with either GE-1 strain (□), AG-83 (○) or
UR6 (Δ) for 2hr (designated as o hr post infection), then cells were washed to remove intracellular
parasites and further incubated at 37°C for 24-72 hr fresh medium for replication.
Results are mean of three different experiments performed with duplicate samples.

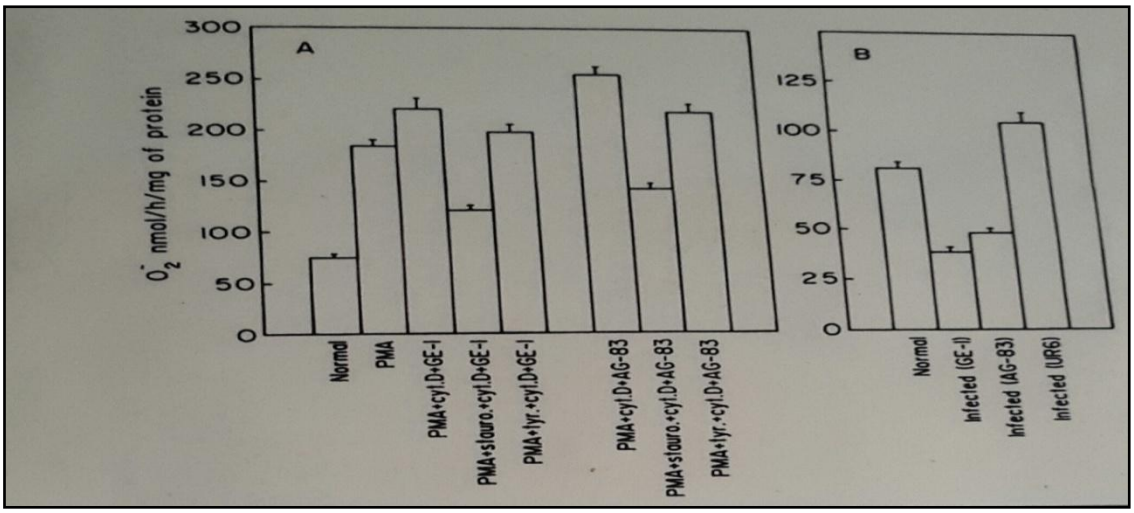

Fig.3 Superoxide generation of macrophages either attached to [A] or infected with *L. donovani* strain[B]. Superoxide production was measured by superoxide dismutase inhibitable
ferrycytochrome C reduction. Macrophages monolayers were prepared, pre-incubated with PMA followed by or not with different kinase inhibitors (staurosporine –stauro, tyrphostin – tyr) as described and then allowed to attached (+Cyt. D) or/then infect for ~48hrs with different strains of promastigotes. In attachment experiment, macrophage was incubated with PMA for 1hr at 37°C and cultured media were collected for superoxide assay. Results are mean ±S.D. of three separate experiments.

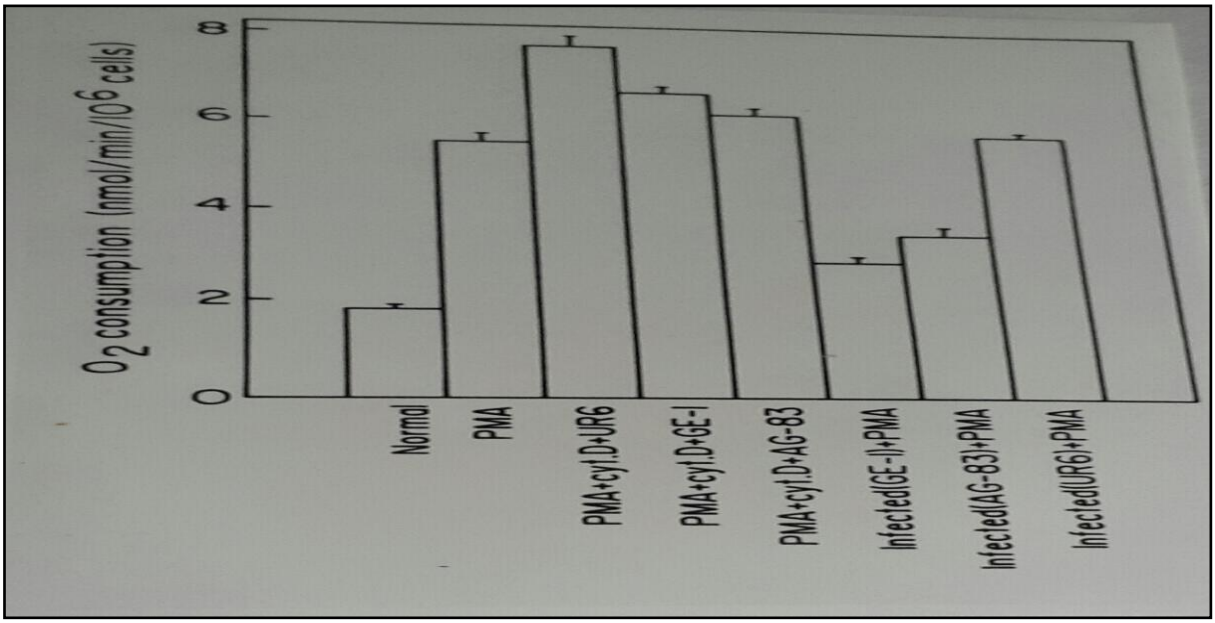

Fig.4 Oxygen consumption of macrophages during attachment or infection with virulent versus avirulent *L. donovani* attached (with Cyt.D) or infected (~48hrs) macrophages (10^6/ml) were incubated in the presence or absence of PMA (1µg/ml for15mins)

Table-I
Effects of PKC and PTK on parasite mediated NO release in murine peritoneal macrophages[1]

CELLS	AGENTS	NITRITE(µmoles)[4]/10^6cells
Macrophages	LPS	80.33±4.31
Macrophages+ Parasite (GE-1)[2]	LPS+Cytchalasin D	110.66±3.10
Macrophages+ Parasite (GE-1)	LPS+Genistein+ Cytochalasin D	45±2.13
Macrophages+ Parasite (GE-1)	LPS+Tyrphostin AG126+Cytochalasin D	40.66±1.21
Macrophages+ Parasite (GE-1)	LPS+Staurosporine+ Cytochalasin D	64±2.41
Macrophages+ Parasite (GE-1)[3]	LPS	29.66±2.6
Macrophages+ Parasite(AG-83)	LPS	44.33±2.91
Macrophages+ Parasite (UR6)	LPS	70.66±3.10

1 The cells were primed with crude IFN-γ (Con A treated T-cells supernatant) and then treated with LPS as indicated in materials and methods.

2 To measure the PKC and PTK mediated NO release during parasite attachment, macrophages were pre-incubated in absence or presence of Genistein, Tyrphostin AG126 or Staurosporine with the indicated dose, washed and parasites were added in presence of Cytochalasin D.

3 Macrophages infected with different strains of parasites, in absence of Cytochalasin D, when at 48hrs, after infection around.8-10parasites/Macrophage for GE-1, 3-4 for AG-83 and virtually no UR6 parasite was detected intracellularly.

4 These values represent the means ± S.D of three separate experiments performed with duplicate samples.

Table-2
Effect of PKC in macrophage membrane protein[1] during interaction and after infection with *L.donovani* virulent strain

Treatment	Histone Phosphorylation (% ^{32}P incorporation/mg of protein) in macrophage membrane fractions (Actual ^{32}P incorporation)
Mouse Peritoneal Macrophage(MPM)	0.21±0.05
MPM+PMA	2.43±0.13
MPM+Cyt.-D+*L.donovani*(GE-1)	1.38±0.03
MPM+Cyt.-	1.25±0.10

D+*L.donovani*(AG-83)	
Macrophage+Cyt.-D +PMA+ *L.donovani* (GE-1)+PMA	2.27±0.19
MPM+Cyt.-D+*L.donovani* (AG-83)+PMA	2.01±0.22
MPM+*L.donovani*(GE-1)	0.32±0.12
MPM+*L.donovani*(AG-83)	0.30±0.17

1 Macrophages were incubated with or without *L.donovani* strain (GE-1or AG-83) (for 2hrs in a cell:parasite ratio of 1:10), in presence or absence of Cytochalasin-D(Cyt.D) (1.5 µg/ml) and with or without PMA(100ng/ml). PKC activities were assayed in membrane fractions.