Alteration In *in -Vitro* Infectivity of *Leishmania donovani* Exposed To Gamma Radiation

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Research Article

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ABSTRACT :
At particular doses of gamma radiation from Co^{60} source suppresses the intracellular parasitism, a fact which raises the question of whether the phenomenon may find practical applications in the outcome of infectious diseases. In this study, stationary phase of promastigotes exposed to radiation doses in the range 10-20 krad (standard dose) resulted in significant parasitization of mononuclear phagocytic system in vitro. However, promastigotes irradiated with 20krad consistently resulted in higher parasitization and optimum infection after acquiring the shape of amastigote-like organism than those exposed to either higher or lower radiation doses. It was observed that 10 krad was necessary to immobilize immediately the organisms, whereas only 30 krad rendered them noninfective and 40-50 krad made promastigotes unable to recognize the binding ligand for attachment to macrophage cell. In comparison to the irradiated parasite, the rate of phagocytosis of 20 krad irradiated cells, were higher while considering the percentage of infected macrophages, the mean number of engulfed parasites by each macrophage cells and the statistics of the two. The rate of infected cells was approximately 8% greater in 20 krad irradiated cells than nonirradiated cells.

Keywords: Intracellular parasitism, gamma radiation, macrophage, phagocytosis

INTRODUCTION:

All *Leishmania* spp., regardless of the disease syndrome, resulting from the infection, parasitize members of the hosts's mononuclear phagocyte system. The problems inherent in exploiting the macrophage fall into three main categories: first, identification and entry into chosen cell type; second, survival within a cell that has evolved to kill invading microbes; and third, long term survival within an antigen presenting cell (Alexander and Russel, 1992). Leishmanial infection of macrophages represents an unique example of intracellular parasitism. Of particular interest in the dilemma in considering the role of macrophages in leishmaniasis: they are both the benefactor or the host cell from parasitological points of view and the executioner or the effector cell in the context of host immunity. In existence must be a delicate balance governed by intricate host parasite interplays between the killing mechanisms of macrophages and the survival mechanisms of *Leishmania*. *Leishmania* species must attach themselves to macrophages before their intracellular entry. The *Leishmania*-macrophage binding is a membrane phenomenon akin to ligand receptor interactions in a broader sense. Expression of this receptor and binding of *Leishmania* to this receptor can be modulated by a variety of agents. The *in vitro* systems presented here offer a way to analyze under controllable conditions of the interesting phenomena involved in the balance of irradiated *Leishmania*-macrophage interactions. Since hamster serves as an excellent animal model for human visceral Leishmaniasis, their macrophages obviously the host cells of choice for such studies (Chang et al., 1986). *Leishmania donovani*-hamster macrophage interactions *in vitro* have been the subject of investigation by several scientists with promastigotes (Miller and Towhy, 1969) or amastigotes (Bhattacharya and Janovy, 1975). Promastigotes bind avidly to macrophages and less well or poorly to lymphocytes. Ameneh et al (2013) observed that the presence of AgNPs in low concentration (which is not toxic for liver and spleen) simultaneous with UVB irradiation in low cumulative dose is effective to treat the *L. major* lesions, and can prevent the visceral course of this disease and reduce the parasite load in spleen in vitro. The present study was undertaken to investigate whether *in vitro* modulation of the *Leishmania* promastigote by gamma radiation affects the attachment and the internalization of irradiated *L. donovani* promastigotes as an attenuated form by macrophages in turn to reduce the parasitic load in spleen. For studying any alteration in binding mechanism which might be potentially effective in designing vaccine, we have used the system of attenuated

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L. donovani promastigotes and hamster peritoneal macrophages *in vitro*.

MATERIALS AND METHODS

Leishmania stock:

Promastigotes of *Leishmania donovani* (MHOM/IN/1983/AG83) were grown at 22^0 C in medium 199 (pH7.4) supplemented with 10% heat inactivated fetal bovine serum, 2mML-glutamine, 100U of penicillin G sodium and 100 g of streptomycin sulfate per ml and subcultured in the same medium at an average density of $2x10^6$ cells/ml.

Radiation exposure:

The late log phase cell culture ($3.7x10^6$cells/ml) was taken for radiation exposure and exposed to a Co^{60} gamma source for irradiation at 23^0 C using doses in the range of 10, 20,30, 40 and 50krad (Gammacell 220) which delivered a dose rate of approximately 12 krads/hr at an exposure distance of 50 cm to the 100% radiation area. The dose rate (12 krad/hr) was measured with Fricke dosimeter (Spinks and Woods, 1976). The irradiated promastigotes were harvested from culture by centrifugation at 125 x g at 4^0 C and taken for the experiment.

Hamster macrophage isolation and culture:

Macrophage cells used were isolated and cultured from peritoneal cavities of three hamsters (weighing 40gm) with prestimulation with 4% thioglycollate broth according to the method described by Chang and Dwyer 1976. HBSS (Hank's Balanced Salt Solution) with 25mM Hepes was injected into peritoneal cavities stimulated two days earlier. Peritoneal fluid was then withdrawn, pooled, centrifuged and resuspended in RPMI 1640 medium. Cell suspension in 0.5ml portions each containing 10^6 cells was distributed to coverslips in petridishes. Cultures were incubated at 37 ± 1^0C in 5% CO_2 in air for 6 hours for cell adhesion and then flooded with additional medium. Before use, coverslips with cell monolayers were rinsed with medium to remove nonadherent cells.

Infection Experiment:

For infection, the hamster macrophages cultured in vitro on cover glasses for 1-7 days and were transferred to three fresh petri dishes. Nonirradiated and irradiated promastigotes were washed twice in RPMI1640. Living promastigotes were counted in a haemocytometer and resuspended in macrophage culture separately. After incubating each set of petridish for one hour under the same conditions, parasite/macrophage culture were taken on coverslips in each case and rinsed with fresh medium to remove free parasites and was prepared for microscopic observations.

Light microscopic study:

Parasite/macrophage cell suspension in case of both treated and untreated sample on coverslips were fixed in methanol and then stained with Giemsa. Infectivity or the percent of infected macrophages containing at least one parasite and endocytic index (Bianco et al, 1975) were dteremined by examining at least 100 macrophages per coverslip respectively.

$$Endocytic\ \text{index} = \frac{Mean\ \text{number of intracellular parasite}}{\text{Infected macrophage}} x\ \%\ \text{of infected macrophage}$$

$$Mean\ \text{number of intracellular parasite} = \frac{\text{Total number of intracellular parasite}}{\text{Total number of macrophage}}$$

Statistical Analysis

The data collected was subjected to statistical analysis and their mean values were calculated. Applied statistics *t*-test with the level of significance set at $p<0.05$ was used to test the significance of differences between the mean values of independent observations using XLSTAT 2010.The results were presented as the mean ± S.D.

RESULTS AND DISCUSSION

Morphologically distinct phagocytic events of macrophages were observed after promastigote binding, including the formation of tubular pseudopodia or cytoplasmic extension devoid of cell organelles in close contact with the parasites and ruffles. In case of nonirradiated promastigote, some macrophages containing promastigotes and other showing partially engulfed promastigotes had been clearly found (Fig.1). The promastigotes were engulfed by their anterior (flagellar) end. A vacuole formed around the engulfed promastigote and the flagellum remained embedded in the cytoplasm of the macrophage. In some cases nonirradiated promastigotes within the macrophage became swollen and assumed an oval shape (Fig. 2). Few promastigotes were seen rarely to have attached by the cell body. 5-6 promastigotes were attached with individual macrophage at a time.

In case of irradiated promastigotes, some notable features had been found. For 10 krad irradiated promastigotes, the phagocytic phenomenon was same as that of nonirradiated promastigotes. Some partially engulfed promastigotes were found. The engulfed promastigotes within the macrophage assumed an oval shape and its density gradually diminished (Fig 3). The vacuole formation in macrophage around the engulfed promastigote was as usual, as that of nonirradiated promastigotes. In case of 20 krad irradiated promastigotes, some promastigotes were engulfed by anterior end and some were by posterior end. There was some competition between the promastigotes for attachment to the macrophage. Distinct tubular pseudopodia of macrophages were found (Fig.4). In case of 30 krad irradiated promastigotes, the flagellum protrudes from cell (Fig.5). The engulfment of promastigotes by macrophages was not found. Macrophage and promastigotes irradiated at this high dose remained side by side in the field as if they could not recognize each other. Pseudopodia initiation was not found, the attraction was not exerted upon the promastigotes. In case of 40 krad irradiated promastigotes, there was no phagocytic event found (Fig. 6). They were morphologically changed to amastigote-like form *in vitro*. For that, engulfment event of macrophage was somewhat modified. There was no attraction between the macrophage and promastigotes. In case of 50 krad irradiated promastigotes, macrophage did not recognize promastigote (Fig.7).

Endocytic index: From figure 8, the basic parameters of the *Leishmania* – macrophage interaction by counting the number of promastigotes and amastigote-like form for 4 hrs incubation after different radiation doses were ascertained and shown in table 1. It revealed that percentage of macrophage infection decreased as the radiation

dose increased at 10 krad but at 20 krad percentage of macrophage infection increased with elevated radiation doses and so also the mean number of parasites per infected macrophages resulting the endocytic index enhances at 20 krad radiation doses. But at 30, 40 and 50 krad, reverse result was found. It indicated that engulfment was highest at 20 krad. The percentage of infected macrophages (30 ± 2.08), the mean number of parasites per infected macrophage (28 ± 0.12) with the endocytic index (84 ± 0.249) were notable in comparison with other radiation doses. At 10 krad, the engulfment was moderate, 16.3% of infected macrophages, whereas the mean number of parasites per infected macrophages was 1.18% and the corresponding endocytic index was 19.23%. At 40 krad, the number of engulfed cells were lowest with very low percentage of viable parasites. Here mean number of engulfment by each parasite and the percentage of affected macrophage were negligible (1.0 ± 0.25, 6 ± 1.5 respectively). So this radiation doses had been proved to be lethal for *Leishmania* parasite. In comparison to the irradiated parasite the rate of phagocytosis of 20 krad irradiated cells, were higher while considering the percentage of infected macrophages, the mean number of engulfed parasites by each macrophage cells and the statistics of the two. The rate of infected cells was approximately 8% greater in 20 krad irradiated cells than nonirradiated cells (Fig.8).

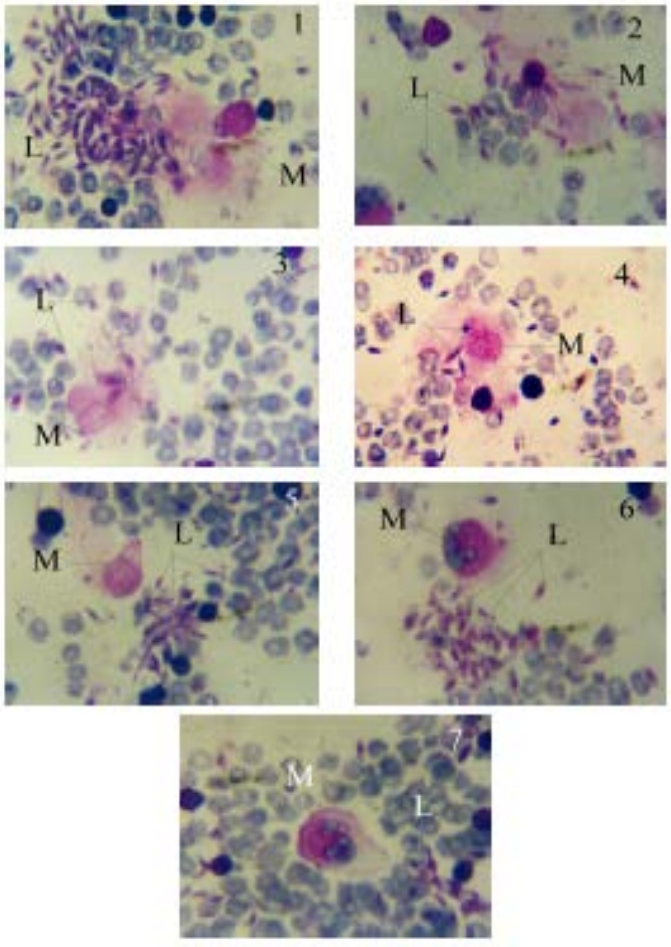

Fig. 1 – 7: Giemsa stained light microscopic photograph on attachment and uptake of nonirradiated and irradiated promastigotes of *L. donovani* (L-*Leishmania*, M-Macrophage) ; (**Fig.1, 2)** non irradiated**; (Fig. 3)** 10 krad ; (**Fig. 4**) 20 krad; (**Fig. 5**) 30 krad; (**Fig. 6**) 40 krad; **Fig. 7**: shows no attachment and uptake of 50 krad irradiated promastigotes by macrophages.

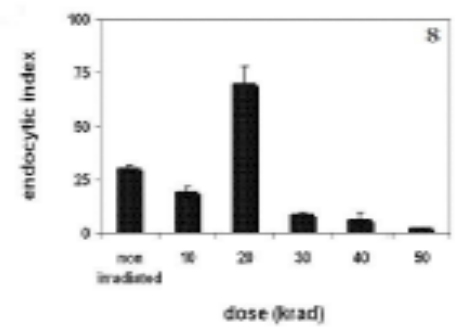

Fig. 8 . Endocytic index of nonirradiated and irradiated *L. donovani*

Table 1: Endocytic index of non irradiated and irradiated *L. donovani*. Results show five individual experiments + S.D.

Radiation dose	Percentage of infected macrophages	Mean number of parasites per infected macrophages	Endocytic index
Non irradiated	22.0 ± 1.98	1.4 ± 0.09	29.9 ± 0.18
10 krad	16.3 ± 2.08	1.2 ± 0.11	19.2 ± 0.23
20 krad	30.0 ± 2.08	28.0 ± 0.12	84.0 ± 0.25
30 krad	8.0 ± 0.67	1.1 ± 0.20	8.4 ± 0.13
40 krad	6.0 ± 1.50	1.0 ± 0.25	6.0 ± 0.36
50 krad	2.0 ± 0.89	1.1 ± 0.25	2.2 ± 0.22

CONCLUSION

Leishmania are a resilient group of intracellular parasites that infect macrophages. *Leishmania* have developed unique adaptive mechanisms to ensure their survival in the harsh environments faced throughout their life cycle. Irradiation of protozoa interferes with their physiological processes and frequently inhibits their normal development and infectivity. Irradiated cells have different levels of radiation injury. It was observed that at particular radiation doses, the formation of amastigote like structure increased. From the present study it was observed that organisms irradiated with 20 krad normally show infectivity *in vitro*; however, if the same organisms were kept in fresh media for about three subsequent subcultures, they became infective in vivo (observed in another experiment). This may be due to postradiation selection of viable and infective organisms. In case of 30-50 krad irradiated promastigotes, the engulfment by macrophages were not found and they remained side by side in the field as if they could not recognize the macrophages. The promastigotes irradiated at these doses were morphologically changed to amastigote-like form *in vitro.*

From the present observation, it was evident that nonirradiated and irradiated cells differ in 3 aspects: (a) counting the number of amastigotes-like structure per infected/noninfected macrophage cells; (b) the infectivity status; and (c) the statistics of endocytic index through the process of phagocytosis (Nogueira and Cohn, 1976). The statistics of endocytic index through the process of phagocytosis showed the basic parameters of *Leishmania* macrophage interaction. This interaction index indicated that the cells irradiated at 20 krad were more infective and virulent and in this case the transformation was complete at 20 krad. So, there may be a correlation of increase or decrease of infectivity with that of radiation doses. But with higher radiation doses there may be some

damage in promastigote cell which desolate the "Zipper" hypothesis of Griffen et al.(1975) and Silverstein (1977) of attachment between promastigote ligand and macrophage receptor. Alexander and Vickerman, 1975 and Rivier et al., 1993 showed that the flagellar tip is the favoured point of attachment in case of normal promastigotes. In usual cases, 5 to 6 promastigotes can adhere to a macrophage and showed the highest ability to attach and penetrate the macrophages. In case of irradiated promastigotes, upto 20 krad radiation doses, the attachment and engulfment phenomenon was same as that in case of nonirradiated promastigotes. But promastigotes exposed above 30 krad were morphologically changed to amastigote-like form and the phagocytic event by macrophages was not found. The flagellum protrudes from the cell. In some cases, the engulfment of irradiated promastigotes occurred through the posterior end. The reasons for this were not known, but it may be related to surface changes and presence of certain ions (Miller and Twohy, 1969).This presume that promastigotes irradiated at higher doses has got changed binding ligands for attachment to mononuclear phagocytes (Lemma and Cole, 1974). Considering the invasion strategy of *Leishmania* parasite the molecules present on the cell surface of promastigotes are of paramount importance as the parasite membrane antigens are the interface between the parasite and its vertebrate and insect hosts. Their altered topography and receptor interactions with host cell decide the acceptability, survival, multiplication, pathogenesis or killing of the parasite in the host (Alexander and Russel, 1992). The mechanism of phagocytosis are dependent upon actin polymerization of flagella and cell membrane protein (Chang, 1979; Rittig et al., 1998). Theses plasma membrane proteins in 30-50 krad irradiated promastigote were damaged. So, it can be presumed that actin polymerization was inhibited and for that the movement of promastigotes towards macrophage was stopped.

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