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Resistance Produced in Golden Hamsters by Inoculation with Ultraviolet-irradiated *Leishmania donovani* Promastigotes

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Abstract

The effect of ultraviolet irradiation (UV) on motility, morphology, reproduction, infectivity, and vaccine potential of *Leishmania donovani* were investigated. Exposure of *L. donovani* promastigotes to UV-irradiation for 20 minutes or longer was necessary to immobilize immediately all the organisms, whereas exposure for as short as 40 seconds prevents them from causing infection. Live promastigotes challenge of hamsters immunized with promastigotes exposed to UV-irradiation for 40 or 50 seconds results in sterile immunity. Hamsters receiving a previous intraperitoneal infection with promastigotes exposed to UV-irradiation for 1—5 minutes showed significantly lower numbers of visceral parasites after challenge than control animals. Promastigotes exposed to UV-irradiation for 10 minutes or longer failed to provide hamsters with any protection against challenge infection. The effect of exposure to UV-irradiation on infectivity of *L. donovani* was first studied.

Key words: Visceral leishmaniasis, UV-irradiation, Immunization, Golden hamster

Introduction

Kala-azar or visceral leishmaniasis is an endemic disease in Iraq (Bashir, 1954; Taj-Eldin and Al-Alousi, 1954; Sukkar, 1986) and about five thousand patients being reported every year (Sukkar, 1985). Ninety percent of the patients are within the first three years of age and from both urban and rural areas (Nouri and Al-Jeboori, 1973; Sukkar *et al.*, 1984).

The caustive agent of this disease is an obligate intracellular protozoan parasite called *Leishmania donovani* which infects cells of the reticuloendothelial system. The organisms transmitted from one vertebrate host to the other by small bitting flies belonging to genus Phlebotomus.

Visceral leishmaniasis in golden hamsters resembles the human disease (Stauber, 1955), so the hamster seems to be a logical experimental mode for studies of acquired resistance.

Although the possibility of producing effective immunization against visceral leishmaniasis has occupied the attention of investigators for long time, only limited success has been achieved in protecting golden hamsters by inoculating them with live organisms intracardially (Stauber *et al.* 1958) or subcutaneously (Farrell, 1976). On the other hand Al-Qubaisi (1980) failed to obtain any protection in hamsters by subcutaneous inoculation with live normal promastigotes to challenge infection. However, Ilardi *et al.* (1974) reported that immunization of hamsters with X-irradiated promastigote of *L. donovani* delays patency of challenge infection, as measured by the appearance of splenic amastigotes.

In view of these consideration and of the promising results obtained in rodents by previous inoculations with UV-irradiated *Trypanosoma rhodesiense* (Charoenvit and Campbell, 1981), *Trypanosoma evansi* (Farag, 1986) and *Echinococcus granulosus* (Molan and Saeed, 1988), the possibility of producing effective immunization in golden hamsters against *Leishmania donovani* by this method was investigated. In addition, the effect of UV-irradiated on motility, morphology and infectivity of *L. donovani* promastigotes to golden hamsters was also investigated. This to our knowledge is

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the first study dealing with the effect of UV-irradiation on *Leishmania donovani.*

Materials and Methods

Source and Maintenance of organisms

A stock of *Leishmania donovani* (BRC 1), isolated in 1982 by bone marrow aspiration from one year old girl (from Baghdad province), was received in 1987 in golden hamster from Scientific Research Council, Baghdad. The organisms were routinely maintained in golden hamsters. For *in vitro* cultivation, we used NNN medium. In order to avoid variation in virulence due to differences in the growth phase, all promastigotes were obtained from the third subculture on the sixth day when the growth was at its peak. Collected promastigotes were pelleted by centrifugation for 10 minutes at 1000 rpm, the supernatant was sucked off and the pellet washed 3 times with sterile Lock's solution and then the pellet was resuspended in fresh Lock's solution. The methods of parasite enumeration in the culture, spleen and liver tissues were as previously described. (Stauber *et al.*, 1958; Stauber, 1962).

Experimental animals

Golden hamsters (*Mesocricetus auratus*) were obtained from the Scientific Research Council, Baghdad. All experimental infections were initiated using male, random-bred hamsters of approximately 8 weeks of age. The animals received water and pellet food *ad lib* and fresh carrots or cucumber once a week.

Irradiation of promastigotes

The source of irradiation was ultraviolet lamp of 2537 A obtained from Phywe company (West Germany). A suspension of $500 \times 10^6 - 600 \times 10^6$ promastigotes in 5 ml Lock's solution in a glass petri dishes with 5 mm depth was exposed (at room temperature, $22 \pm 2°C$) to UV-irradiation at a distance of 5 cm from the light source for varying lengths of time. The distance was kept constant for the exposure in all the experiments. The UV-tube was switched on for 10 minutes before the exposure of promastigotes in order to obtain even emission radiation.

Experiment 1.

Effect of UV-irradiation on motility of promastigotes *in vitro*. Promastigotes were exposed to UV-irradiation for varying lengths of time (2, 4, 6, 8, 10, 12, 14, 16, and 20 min). Samples of 0.1 ml (100—200 promastigotes) were removed from the experimental petri dishes, and examined under the microscope 1, 2, 4, 6, and 8 hours after irradiation. The number of promastigotes immobilized by irradiation is expressed as a percentage of the total number in the sample.

Experiment 2. Effect of UV-irradiation on reproduction of promastigotes *in vitro*.

Seven batches of promastigotes were dispensed in petri dishes, each of which contained 10×10^6 promastigotes per 3 ml of sterile Lock's solution and exposed to UV-irradiation for 0, 1, 2, 3, 5, 10 and 20 minutes respectively, and then the irradiated promastigotes were transferred into fresh NNN media (3 vails for each exposure time). The cultures were left at incubator (25°C) and examined once every two days for reproduction and the results are shown in Fig. 1.

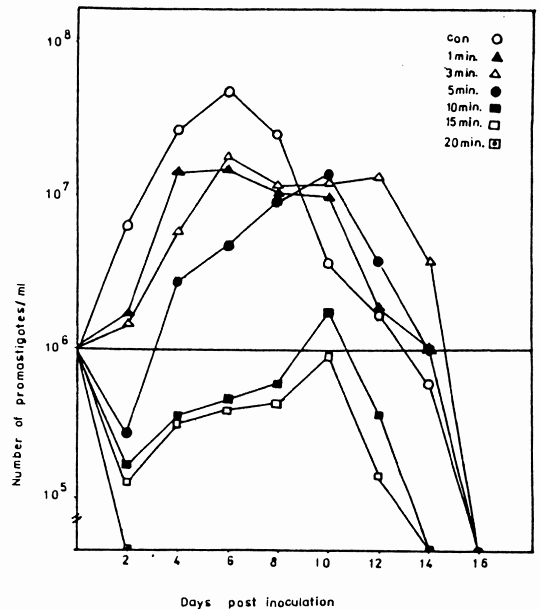

Fig. 1. Population growth of control and UV-irradiated *Leishmania donovani* in NNN medium at 24°C.

Experiment 3. Effect of UV-irradiation on infectivity.

Twenty, 8—10 weeks old male golden hamsters were allocated into five equal groups. The animals in the first four groups were infected with 100 × 10^6 promastigotes which had been exposed to UV-irradiation for 10, 20, 30 and 40 seconds respectively, while those of the fifth groups were infected with the same number of normal non-irradiated promastigotes to serve as controls. Forty days later, all the animal were sacrificed and the weights of spleen, liver and spleen length were recorded for each animal and impression smears were prepared from the spleen and liver of each animal and stained with Giemsa stain. The results of this experiment are shown in Table 2 and Fig. 2.

Experiment 4. Evaluation of the protection induced by UV-attenuated *L. donovani* promastigotes in hamsters

Forty eight male hamsters were divided into eight equal groups. The animals of first group injected intraperitoneally with 100 × 10^6 promastigotes which had been exposed to UV-irradiation for 40 seconds. Those of the other groups were injected with the same number of promastigote which had been exposed to UV-irradiation for 50, 60, 70, 100 seconds, 5, 10, and 15 minutes. Twenty days later, all 48 hamsters, together with 6 previously uninfected hamsters, were challenged, *per os*, with 20 × 10^6 normal promastigotes and dissected 40 days after challenge. We used the mean number of splenic amastigotes as the sole parameter for evaluation of the vaccine potential of UV-attenuated promastigotes. The results of this experiment are shown in figure 3.

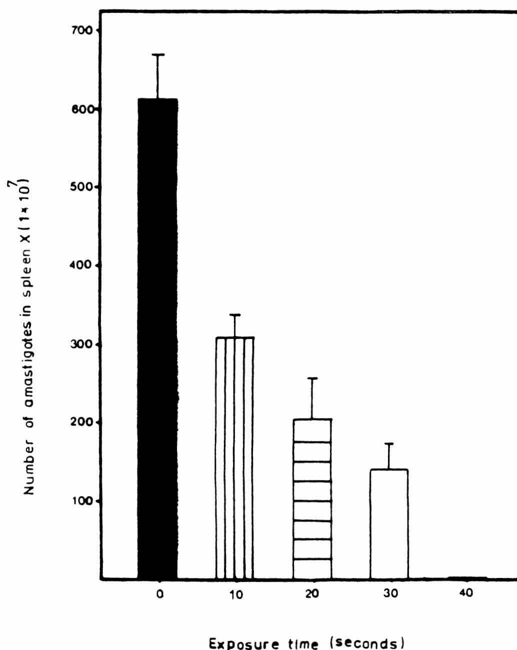

Fig. 2. Spleen parasitization during infection with *Leishmania donovani* in control hamsters (■) and hamsters infected with promastigotes exposed to UV-irradiation for 10 (▥), 20 (⊟), 30 (□) and 40 (—) seconds.

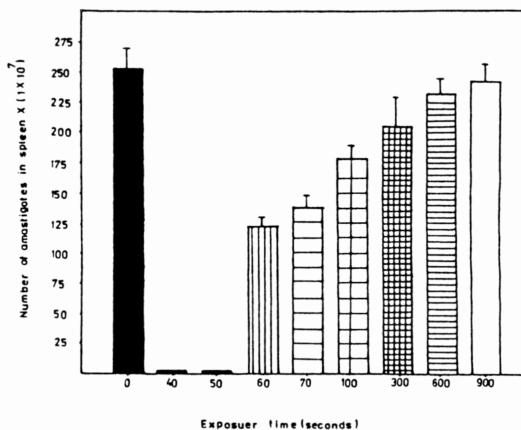

Fig. 3. Spleen parasitization during a challenge *Leishmania donovani* infection in control hamsters (■) and hamsters previously infected with promastigotes exposed to UV-irradiation for 40 (—), 50 (—), 60 (▥), 70 (⊟), 100 (⊞), 300 (▦), 600 (⊟), and 900 (□) seconds.

Results

Experiments 1 and 2.

It can be seen from Table 1 that exposure of the promastigotes to UV-irradiation for 20 minutes immobilizes all organisms immediately, but 6 hrs were required to do similar effect when the promastigotes were irradiated for 8 minutes. Shorter exposure times had progressively slower

Table 1. Effect of ultraviolet irradiation on motility of *Leishmania donovani* promastigotes *In vitro*

Exposure time (min)	% Immobilization at variots times after irradiation				
	1hr	2hr	4hr	6hr	8hr
control	0	10	35	70	85
2	0	10	40	73	95
4	0	15	60	90	100
6	5	18	70	94	100
8	5	30	82	100	100
10	12	50	84	100	100
12	14	55	87	100	100
14	18	90	100	100	100
16	25	95	100	100	100
20	100	100	100	100	100

and less severe effects.

The effect of UV-irradiation on the reproduction of promastigotes is presented in Fig. 1. Organisms exposed to UV-irradiation for 3 min or less reproduced and gradually achieved high population densities. The slight increase in population seen in organisms exposed to UV-irradiation for 5 min or longer may be either due to completion of divisions that had started prior to irradiation or due to the reversion of the radiation damage. All organisms exposed to 15 min or longer lost their ability to reproduced. Morphological abnormalities in the irradiated organisms were observed and their number was proportional to the radiation dose: the longer the exposed time, the more abnormalities. The abnormalities seen included shrinkage, rounding, ballooning, loss of flagella, possession of two or more flagella, multinucleation, and vaculation.

Experiment 3.

It can be seen from Table 2 that the intraperitoneally inoculated hamsters with promastigotes exposed to UV-irradiation for 30 seconds or longer showed significantly ($P < 0.01$—0.001) lower weights in liver and spleen, and significantly ($P < 0.01$—0.001) less in spleen length than their counterparts infected with non-irradiated promastigotes. Exposure of promastigotes for 40 seconds to UV-irradiation renders them non infective to hamsters, as no amastigotes have been seen in the impression smears prepared from the liver and spleen of these animals and no promastigotes have been found in the cultures incubated at 25°C which had been inoculated with biopsied liver and spleen of these animals. These animals also, showed approximate spleen and liver weights and spleen length to the same organs of the non-infected counterparts. Student's "t" test indicated that the mean number of splenic amastigotes in the hamsters infected with promastigotes irradiated for 10, 20, or 30 seconds were significantly different from the controls, infected with normal non-irradiated organisms ($P < 0.001$).

Table 2. The effect of ultraviolet irradiation on the average spleen, liver weights and spleen length of hamster infected intraperitoneally with 100×10^6 *Leishmania donovani* promastigotes and killed (40) days post infection

Exposur time (sec)	No. of animal	Mean liver wt. (gm) ±SD	Mean spleen wt. (gm) ±SD	Mean spleen length (cm) ±SD
control	4	6. 25±0. 46	0. 19±0. 04	3. 3±0. 44
10 Sec.†	4	5. 62±0. 53	0. 16±0. 05	3. 14±0. 43
20 Sec.*	4	5. 45±0. 23	0. 12±0. 024	2. 6±0. 17
30 Sec.*	4	5. 24±0. 29	0. 11±0. 0088	2. 3±0. 23
40 Sec.*	4	5. 22±0. 46	0. 10±0. 05	2. 2±0. 34

* $P<0.05$—0.001 V control by student's t-test.

† Not significant V control.

Experiment 4.

As the previous experiments showed that the shortest exposure time that renders the promastigotes non-pathogenic while they are still active and can reproduce *in vitro* is 40 seconds, therefore, 40 seconds—15 minutes was considered to be the attenuating exposure range. All the hamsters inoculated intraperitoneally with a single dose of 100×10^6 promastigotes which had been exposed to UV-irradiation for 40 or 50 seconds, were able to develop sterile immunity against a challenge infection of 20×10^6 live non-irradiated promastigotes as evidenced by the disappearance of splenic amastigotes in the impression smears prepared from the spleen of these animals.

Hamsters immunized with promastigotes exposed to UV-irradiation for 60, 70, 100 seconds and 5 minutes, and challenged 20 days later with normal promastigotes had mean spleen parasite burdens of 122.66 ± 6.9, 137.2 ± 11.4, 178.3 ± 11.39 and 229.4 ± 12.6 respectively (Fig. 3). The difference in spleen parasite burdens of these animals and control hamsters (252.7 ± 17.8) was statistically significant (Student's t-test $P < 0.02 - 0.001$). The mean spleen parasite burdens of hamsters immunized with promastigotes exposed to UV-irradiation for 10 and 15 minutes (234.2 ± 12.3 and 242.4 ± 14.5 respectively) and challenged with normal promastigotes, was not statistically different from those of the control non-immunized animals ($P > 0.05$).

Discussion

From the results it appears that exposure of the promastigotes of *Leishmania donovani* to UV-irradiation for 20 minutes immobilizes all the organisms immediately, while promastigotes exposed to UV-irradiation for 15 minutes or longer failed to reproduce in the culture. The damaging effect of increasing time of exposure to UV-irradiation seen in the present study is similar to that reported by many authors on other parasite species (Stankieweiz *et al.*, 1970; Lemma and Cole, 1974; Ghandour and Webbe, 1975; Bezubik and Wedrychwicz, 1976; Molan, 1983; Molan and Saeed, 1988).

Exposure of *L. donovani* promastigotes for as short as 40 seconds to UV-irradiation prevents the organisms from causing infection to golden hamsters. Similarly, Charoenvit and Campbell (1981) reported that exposure of *Trypanosoma rhodesiense* to UV-irradiation for one minute renders the trypanosomes non-pathogenic. Farag (1986) demonstrated that exposure of *Trypanosoma evansi* to UV-irradiation for 30 seconds abolishes the infectivity of these organisms to mice.

The present results revealed that the promastigotes exposed for 40 or 50 seconds to UV-irradiation were very actively motile but non-pathogenic to golden hamsters and conferred 100% protection against the challenge infection of normal non-irradiated promastigotes. Similarly Charoenvit and Campbell (1981) found that immunization of mice with *Trypanosoma rhodesiense* exposed to UV-irradiation for 2, 5, or 10 minutes results in sterile immunity. Although it is difficult to compare different host parasite system, our result's also agree with those of Katiyer *et al.* (1968) who observed that subcutaneous injection of *Nippostrongylus brasiliensis* which had been exposed to UV-irradiation, gave complete protection against a challenge dose of normal larvae. Alexander (1982) and Howard *et al.* (1982) have made use of gamma irradiated organisms both promastigotes and amastigotes of *Leishmania tropica* or *L. major* in the mouse and obtained marked protection to reinfection. It should be noted that Lemma & Cole (1974) attempted but failed to obtain protection with UV- and gamma irradiated *Leishmania enrietti* in the guinea pig.

The absence of visceral parasites in hamsters immunized with promastigotes exposed to UV-irradiation for 40 or 50 seconds and the significant differences in visceral parasites levels between other immunized and control hamsters suggest the possibility that immunized hamsters possess activated macrophages in their visceral organs such as are known to occur during infection with other intracellular organisms (Mackaness and Blanden, 1967; Mackaness,

1969). Furthermore, Howard *et al.* (1982) in a study of susceptible mice which they protected by immunization with irradiated organisms, found that only humoral responses were stimulated by the inactivated organisms. They also mentioned that the cell-mediated immunity did appear, but only after challenge with living organisms.

Previous attempts at immunization against *L. donovani* in the hamster have yielded primary negative results (Stauber *et al.*, 1958; Al-Qubaisi, 1980) or partial immunity (Farrell, 1976). In these studies, an intracardial or subcutaneous route of inoculation of live amastigotes or promastigotes was used a method of attempted immunization. It is probable that the direct intravascular or subcutaneous administration of parasites does not induce or induce weak acquired immunity, especially if cell-mediated immunity, rather than humoral immunity plays a role in protection against visceral leishmaniasis. In addition, a severe challenge may overcome a weak immunity which would be sufficient to enable the test animals to withstand a milder challenging dose.

UV-irradiation seems to be more effective than X-irradiation as immunization of golden hamsters with X-irradiated *L. donovani* promastigotes (Ilaradi, *et al.*, 1974) only delays the patency of challenge infections, while in our study immunization of hamsters with promastigotes exposed to UV-irradiation for short periods lead to the development of sterile immunity against challenge infection with normal promastigotes. X-irradiation may causes severe damage or kills the promastigotes. It appears that the organisms must survive and react with the hosts cells for considerable time before the host acquires detectable protective cellular immunity (Bray and Bryceson, 1968; Bryceson *et al.*, 1970; Lemma and Cole, 1974). In these studies, both the heat-killed and radioattenuated organisms may die out before the host has enough time to build up protective immunity. Similarly, in the present study we found that hamsters immunized with promastigotes exposed to UV-irradiation for 15 minutes failed to build up protective immunity against challenge infection. It may expected that for extremely high radiation dose, immunogenicity may be damaged. However, Duxbury and Sadun (1969), working with *T. rhodesiense* at very high radiation doses, found that high radiation doses produced less effective immune response than lower doses. Kuhan and Cassida (1981) showed that the use of living parasites of *T. cruzi* (untreated or attenuated with mitomycin c) proved most effective in inducing cytophilic antibodies than use of killed parasites. Coutinho (1955) and Kretschmar (1965) tried but failed to vaccinate quinea pigs with heat-killed promastigotes of *Leishmania enriettii*.

In conclusion, the present study indicates that the golden hamster can exhibit acquired resistance to an intraperitoneal induced infection with *L. donovani* promastigotes through previous exposure to intraperitoneal infection with the same organisms which had been exposed to UV-irradiation for various periods.

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