EFFECT OF IMMUNOMODULATOR DARAPRIM ON POTENTIATION OF VACCINE PROTECTION OF LEISHMANIA MAJOR IN BALB/c MICE

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ABSTRACT

In this study, a crude leishmanial antigen, was prepared by sonicating Leishmania major promastigotes used to induce immunity in BALB/c mice against cutaneous leishmaniasis. Correlation between the route of antigen injection and the efficacy of induced protection was examined. To enhance the effectiveness of the antigen, an immunostimulant drug, daraprim (pyrimethamine), was administered simultaneously with the antigen. The experiment demonstrated that simultaneous intraperitoneal injection of the antigen and daraprim resulted in protection from subsequent development of cutaneous lesions. Results of lymphocyte proliferation from mice immunized with either the antigen or antigen-daraprim mixture showed a significant response to the antigen. The results suggest that daraprim can be used in prophylaxis programs to enhance the effectiveness of vaccines for cutaneous leishmaniasis.

Keywords: Leishmania major, Leishmaniasis, Daraprim.

INTRODUCTION

Leishmaniasis, caused by the intracellular protozoan Leishmania sp., is a spectrum of diseases varying in severity from the fatal visceral form to a self-healing cutaneous lesions. It infects twelve million people worldwide(1). Cutaneous leishmaniasis (CL) is caused by L. major which lives in mononuclear phagocytes and control of the infection depends on an adequate activation of the infected macrophages to kill parasites and inhibit their replication(2). Results of experimental animal indicate that cure from cutaneous disease is associated with generation of T helper 1 (Th1) cells and the resistance and susceptibility of inbred strains of mice to CL is determined by the balance status between type1/type2 lymphocyte cells(3). Healing of
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Lesions require induction and expansion of Th1 cells which produce IFN-γ, a crucial activator of inducible nitric oxide synthase (iNOS) (3-5). The importance of nitric oxide-dependent killing of intracellular parasites has been demonstrated and it has been shown that iNOS- deficient mice with a resistance background develop non-healing cutaneous lesions(6). In contrast, IL-4 produced by Th2 (Th2) cells leads to disease exacerbation because IL-4 inhibits the expansion of nitric oxide synthase (iNOS) (7). In the highly susceptible BALB/c mice along the activation of Th2 pathway, an early peak of interleukin 4 (IL-4) production in draining lymph nodes occurs by 1 day after infection which results in subsequent development of Leishmania-specific CD4⁺ T cells. This early IL-4 production is not seen in resistant mice and is thought to determine the course of infection (8).

To date it appears that not only healing but also protection using defined antigens is dependent on the induction of Th1-type immune response. The stringent challenge for a protective vaccination in BALB/c mice is conversion of the strong Th2 response to a protective response. Vaccination strategies should clearly aim to produce Th1-promoting cytokines such as IL-12 at the time of cellular response to antigen (9). Some vaccination approaches which have resulted in various degrees of protection in mouse models of infection include: DNA vaccination (10), S.C injection of live or attenuated L. major (11), use of Salmonella (12) or BCG (13) vectors expressing Leishmania antigen, antigen-containing liposomes (14), ISCOMs (15), use of IL-12 and use of Cryptococcus parvum as an adjuvant (16).

There is a wide variation in the availability and efficiency of drugs for the therapy and prophylaxis of parasitic disease in both human and domestic animals (17). Available drugs are ineffective because of low efficiency, high toxicity or the requirement of long courses of pretrial administration. However, some drugs such as liposomal amphotericin B, amphotericin B-arabino-galactan and fungizone have been demonstrated to be effective against Leishmania promastigotes and amastigotes in vitro and against L. major infection in BALB/c mice (18).

In our study the experimental L. major infection in BALB/c mice which has proven to be an extremely valuable model for the study of immunity in cutaneous leishmaniasis was used. In this model, immunomodulatory effect of a drug, daraprim, and its mixture with a leishmanial crude antigen prepared from L. major on the outcome of the cutaneous lesion produced after challenge was studied.

MATERIALS AND METHODS

Animals and parasite

Inbred BALB/c mice were obtained from Pasteur Institute, Tehran, Iran and bred on a pure BALB/c genetic background. Female mice 6-8 weeks old were used throughout.

L. major amastigotes were obtained from human ulcer in Isfahan, Iran and were maintained by cutaneous passage in BALB/c mice. Amastigotes were obtained from lesions of BALB/c mice that had been s.c. inoculated at the base of their tails with 1 x 10⁶ stationary-phase promastigotes in 0.1 ml PBS (pH 7.2). The lesions were excised and infected tissue was completely broken up in RPMI 1640 (Sigma) to free the amastigotes. The cell suspension was centrifuged at 1000 x g to remove debris and then at 2000 x g to sediment amastigotes. The parasites were then inoculated in RPMI 1640 supplemented with 15% heat-inactivated fetal calf serum (Gibco), gentamycin (50 (μg/ml) and penicillin-streptomycin (100 U/ml and 100 (μg/ml, respectively) or were grown in RPMI 1640 overlying NNN medium (14 g agar, 6 g NaCl, and 900 ml distilled water). The promastigotes were not maintained in vitro for longer than 2 months prior to infection. Parasites were passed at intervals in BALB/c mice to ensure that virulence was maintained.

Parasite antigen and drug

For antigen preparation, L. major log-phase promastigotes were washed three times in PBS pH 7.2 and then resuspended in the same buffer to make 4 x 10⁷ cells/ml. The suspension was then subjected to 5 cycles (5 x 1 min) of ultrasonication (MSE Ultrasonicator) for 90 secibels while being cooled in an ice bath. The extract was centrifuged at 1000 x g for 10 min at 4°C to sediment intact cells and nuclei and the supernatant was used in
experiments after its protein content was determined by Lowry method. The antigen preparation was either used immediately or frozen at -70°C and reconstituted at room temperature prior to injection.

Daranprim (pyrimethamine) was purchased from Wellcome Co. (England) and a dose of 3.5 mg/kg was used throughout. To monitor possible direct effect of the drug on the parasite, 12 vials containing 1.5 x 10^6 promastigote cells/ml were set in triplicate and to each triplicate, 1, 3 or 4 mg/kg daraprim (equivalent to the drug dose for a 25 g mouse) was added. The number of promastigote cells was measured at 2 and 4 days after culture.

**Immunization and challenge**

To examine the stimulatory effect of daraprim on the protective immune responses of mice injected with the antigen, four groups each comprising 4-6 female mice of 6-8 weeks old were set. Animals were classified into four groups,

- **Group I**, Ag SC, animals were injected in three successive weeks with an aliquot of 0.1 ml of parasite antigen (containing 18 μg protein) via SC route.
- **Group II**, Ag IP animals were injected in three successive weeks with an aliquot of 0.1 ml of parasite antigen (containing 18 μg protein) via IP route.
- **Group III**, Ag+daraprim, animals were injected in three successive weeks with an aliquot of 0.1 ml of parasite antigen (containing 18 μg protein) via IP and daraprim (5 mg/kg) intraperitoneally.
- **Group IV**, Control, receiving saline only ten days after the last injection, all animals were challenged subcutaneously with 1 x 10^6 promastigote cells in 0.1 ml at the base of their tails. Cutaneous lesions produced were weekly monitored by measuring the diameter of the infected area with a Vernier caliper.

**Lymphocyte proliferation assay**

Eighteen BALB/c mice were set in three groups and injected in two successive weeks as follows.

- **Group I** was injected with 0.1 ml of the antigen (protein content 18 μg) intraperitoneally.
- **Group II** was injected with an antigen- daraprim mixture (18 μg and 3 mg/kg, respectively).
- **Group III** was injected with daraprim only, 3 mg/kg.

Ten to fifteen days after the last injection, all animals were sacrificed and their spleens obtained as the source of lymphocytes for in vitro analysis. Lymphocytes were obtained by gentle homogenization of spleen in RPMI 1640 containing 15% fetal calf serum. The cells were then pelleted at 2000 x g at 4°C and red blood cells lysed with 0.87% NH4Cl. The cells were then washed and their concentration made to 4 x 10^6 cells/ml in culture medium containing RPMI 1640, 2 mM L-glutamine, 25 mM HEPES, 10% fetal calf serum, 50 μg/ml gentamycin, penicillin-streptomycin at 100 U/ml and 100 μg/ml, respectively. A 100 (μl aliquot containing 4 x 10^5 cells/well was then mixed with 100 (μl parasite antigen in triplicate wells of a 96-well flat-bottomed microtiter plate (Costar, Cambridge). For base lines (control) 100 (μl of the medium was used instead of the antigen. The plate was incubated at 37°C in a CO2 incubator for 4-5 days. At the fifth day after incubation, 0.5 μCi/well tritiated thymidine (³H-TDR) was added to each well. Eighteen hours later, the cultures were harvested using an automatic cell harvester and ³H-TDR uptake was determined with a β-counter (Bekman). Blastogenic responses were calculated by stimulation index (SI) which is the proportion between the increases in counts per minute of the test lines to that of the base line.

**Statistical analysis**

Significance was determined by Mann-Wittney and Kruskal-Wallis tests with a value of P (0.05 considered to be significant).

**RESULTS**

**Parasite antigen and drug**

The protein content of the supernatant from sonicated promastigotes (4 x 10^7 cells/ml) was 183 μg/ml. Counting number of parasite at days after their exposure to the drug indicated that no significant difference between mean number of promastigotes/ml in the vials subjected to 0, 1, 3 and 5 mg/kg daraprim occurred by 2 and 4 days after culture (Table 1). This indicates that function of the drug was not due to its direct effect on the parasite.
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Table 1. Mean number (cell/ml) of promastocytes in 0–4 Days after culture

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<th>Daraprim (mg/kg)</th>
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Immunization and challenge

Results of time-course study on the lesion development in animals from different groups is given (Figure 1). The results indicated that s.c. injection of the antigen led to significant lesion enlargement. Although some lesion enlargement emerged from i.p. injection of the antigen, but it was not significant as compared to control group

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![Graph showing lesion diameter over time](image)

Fig. 1. The size of leishmania lesion in the animals pre injected with leishmania s.c.

(Fig. 2). This indicated that i.p. injection of the antigen did not increase the severity of the disease. Disease severity and lesion enlargement were highly reduced when mice were injected intraperitoneally with the antigen and daraprim simultaneously, indicating stimulatory effects of daraprim on anti-leishmanial immune responses of BALB/c mice.

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![Graph showing lesion diameter over time](image)

Fig. 2. The size of leishmania lesion in the animals pre injected with leishmania i.p.

Lymphocyte proliferation assay

The results indicated a significance difference between the stimulation index of the control group (SI±SEM = 1.47±0.45) and the other two groups inoculated with the antigen (SI±SEM = 6.68±2.98) or the mixture of antigen-daraprim (SI±SEM = 7.76±3.1). This observation indicates that i.p. injection of antigen or antigen-daraprim mixture has significantly stimulated T cells responses.

DISCUSSION

In an immunostimulatory assessment such as this work several factors must be taken into consideration. The first issue is the planned route of injection. The results presented here indicated that s.c. injection of leishmanial antigen induced a counter-protective effect on BALB/c mice leading to exacerbation of disease. So far, few reports on successful immunization of animal models using crude leishmanial antigens have been obtained. Even, s.c. injection of leishmanial crude antigens have frequently resulted in disease exacerbation (19-24). In contrast, i.p. injection of leishmanial antigens have been demonstrated to stimulate a protective status against cutaneous leishmaniasis. This was, however, obtained only if the
concentration of the challenge inoculum was lower than 10^5 cells/ml (19,22).

The presented results also indicate that daraprim contains immunomodulator(s) that modulate the infection with L. major. This is confirmed by our previous results demonstrating that daraprim enhanced delayed type hypersensitivity and antibody response(25). A correlation between positive DTH response and healing forms of cutaneous leishmaniasis has been demonstrated while in non-healing forms disease have been associated with negative DTH response (26-27). During the Iraq-Iran war, the health authorities of Iran implemented a prophylaxis program for all soldiers, which included immunization with L. Major and oral administration of pyrimethamine for treatment of malaria. We have noticed that when soldiers were injected with L. Major vaccine (attenuated L. major) intradermally and received pyrimethamine orally, the size of the lesion following vaccination decreased significantly as compared with those of the people in endemic area receiving L. Major vaccination alone.

The timing of antigen and drug administration has been considered an important factor in our model. Most reports indicated a maximum suppression of immune parameters when antigen was administered before or at the time of antigen administration. Further studies on the mechanism of pyrimethamine modulatory action are necessary to clarify its mechanism of therapeutic action in the treatment of Leishmaniasis.

REFERENCES

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