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Partially Induced Protection by a Fraction of *Leishmania Major* Promastigotes against Murine Leishmaniasis

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ABSTRACT

Partially purified antigenic fractions of *Leishmania major* promastigotes were obtained by sodium dodecyl sulfate (SDS-PAGE) and electro-elution. Three isolated protein fractions designated as fractions (Fr.) 1, 2, and 3 correspond to 40-60, 60-80, 80-100 kilodalton (kDa) respectively. They were used for immunization of BALB/c mice against Leishmaniasis. The effects of these fractions on immune response of BALB/c mice against leishmanial infections was investigated by studying the infection course in infected mice, delayed type hypersensitivity (DTH) skin test, lymphocyte proliferation assay (LTT) in them. Subcutaneous immunization of mice with fraction 2 in conjugation with Complete Freund's Adjuvant (CFA) developed partial immunity against *Leishmania major* infection, and induced specific DTH response. Meanwhile this fraction exhibited no exacerbating effect on leishmanial infection course. Subcutaneous immunization with fraction 1 also induced partial protection in lesser extent than fraction 2 against leishmanial infection. *Iran. Biomed. J. 2: 27-32, 1998.*

Keywords: Leishmania major, promastigotes, DTH, leishmaniasis.

INTRODUCTION

Leishmaniasis is a protozoal disease with a spectrum of clinical manifestation in human ranging from self-healing cutaneous to fatal visceral infections. These infections were reported at least from 74 countries around the world [1]. The solid immunity following recovery from cutaneous leishmaniasis correlates with the development of *leishmania* specific CD4 cells of Thl-type. DTH responses to leishmanial antigen (leishmanin test), develops in leishmaniasis patients and their lymphocytes proliferate in response to *L. major* antigen *in vitro.* In the contrary, induction of Th2-type cells will lead to severe pathology in them [2].

Early clinical observations that spontaneously or drug-cured cases seldom show a recurrence of disease, encouraged the belief that development of a prophylactic vaccine is feasible [1]. The identification of antigens that elicit immune responses is an important step toward understanding the immunology of *Leishmania* infection and ultimately in the development of a vaccine. Few immunization strategies are held through the controlled induction of a lesion in an aesthetically acceptable site of the body with live promastigotes or crude parasite extracts [3]. The surface protease gp63, the surface antigen gp46/M2 and the related parasite surface antigen 2 (PSA-2) are some of the antigens inducing significant protection in animal models, when delivered with adjuvant which may not be appropriate for use in humans [2]. Most of the subunit vaccines have been focused on protein antigens. They are easy to identify, isolate, clone and study. If any appropriate antigenic fraction of the parasite could be able to induce a proper immune response, an effective vaccine can be made on its basis [3].

For several years murine model of cutaneous leishmaniasis have been used to evaluate experimental vaccines against *leishmania* parasites. Availability of this experimental models, provides a firm foundation to design studies for identification of immunogens and correlation of specific immunologic responses and protection [4,5]. Present work is an attempt to determine immunogenic fractions of *L. major* promastigotes isolated by SDS-PAGE and electro-elution and assessing their protective effects on immunized BALB/c mice against leishmanial infection.

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MATERIALS AND METHODS

Animals and parasites. Female BALB/c mice 8-9 weeks old were obtained from "Animal Breeding Center", Pasteur Institute of Iran. *Leishmania major* (MRHO/IR/75/ER) originally provided by Dr. E. Javadian, School of Public Health, Tehran, Iran.

Culture media: For parasite cultivation , the parasites were grown at 25° C in RPMI 1640 medium (Sigma) supplemented with 20% heat-inactivated fetal calf serum (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultivation of mononuclear cells were carried out in RPMI 1640 medium supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (Sigma), 100 U/ml penicillin, 100 µg/m1 streptomycin, 2 mM L-glutamine, and 0.1% (v/v) 2-mercaptoethanol.

Preparation of antigens and fractions: Approximately 6 x 10^9 *L. major* promastigotes were collected at the beginning of stationary phase of growth after 6 or 7 subcultures, and washed twice in phosphate buffered saline (PBS) pH 7.2. Promastigotes were resuspended in PBS at the concentration of 4 × 10^8 parasites per ml and they were mixed 1:1 with sample buffer [0.06 M Tris-HC1, pH 6.8, 20% sucrose, 4% sodium dodecyl sulfate, 10% (v/v) 2-mercaptoethanol], boiled for 3 minutes and they were electrophoresed on 10% SDS polyacrylamide gel (3 mA per well). The gels were then cut into 3 horizontal strips [40-60 kDa (Fr.1), 60-80 kDa (Fr.2), 80-100 kDa (Fr.3)] based on the standard curve of molecular weight marker which had been run in parallel. The gel strips were electroeluted (Bio-Rad 422-electroeluter) and the protein content of each strip was derived [6, 7]. In order to remove the SDS residue from these fractions, the lower chamber of electro-eluter was filled with electrode buffer without SDS (Tris-HC1 pH 8.9, glycine). Total protein concentration of each fraction was measured by Lowry method [8], and they were frozen at -70° C.

Preparation of freeze and thawed (FT) leishmanial antigen. Parasites were suspended in phosphate buffered saline and the FT antigen was prepared by ten successive freezing (-196° C) and thawing (37° C) steps.

Immunization Protocol. In this study, 9 groups each containing 19 mice were used for immunization. Each mouse was injected subcutaneously (s.c.) or intraperitonealy (i.p.) with 30pg protein equivalent from one of the fractions mixed with equal volume of CFA (Sigma). Control groups received either eluates from gels which had not been loaded with *leishmania* promastigotes or CFA alone. On the day 10 and 20 after the first injection, the immunized mice received one other injection consisting of one of the antigenic fractions without adjuvant as booster dose.

Inoculation of mice. Ten days after the last immunization mice were inoculated at the base of their tails (s.c.) with 2 × 10^6 virulent parasites obtained at the beginning of the stationary phase of growth after 3 subcultures. The mice were examined weekly and after the appearance of any lesion, diameter of the lesions were measured.

Assessment of Delayed type hypersensitivity (DTH). DTH was determined by skin testing on mice footpad. Each mouse was injected with 50 µ1 of FT Leishmanial antigen (2 × 10^8 parasites per ml) and footpad thickness was measured before testing and also 24, 48 hours after injection. The skin testing was performed ten days after challenging of mice with virulent *L. major* promastigotes.

Lymphocyte proliferation assay. Two weeks after the last immunization, three mice of each group were sacrificed. Their lymph nodes (LN) were removed and gently disrupted with forceps and were forced through a wire mesh. The suspension of isolated cells were washed three times with RPMI medium and were resuspended in CTCM (Complete Tissue Culture Medium) (2.5 x 10^6 cells/nil). Cells were dispensed in 96-well flat-bottomed plates in 200 µl volume. FT leishmanial antigen (7.5 x 10^6/ml) with various concentrations or phytohemagglutinin (PHA) 5 µg were added in triplicate to the wells of lymphocyte culture. The plates were kept in a CO_2 incubator (5% CO_2) at 37°C for 5 days, 18 hours before the termination of culture, cells were pulsed with 1pCi [^{3}H] thymidine (Amersham), and they were harvested on to glass fiber filter paper (Wallace). Incorporation of [^{3}H] thymidine was assessed by liquid scintillation using Beta-counter (Pharmacia).

RESULTS

DTH responses: DTH reactivity was measured by skin test of mice in footpad. All unchallenged immunized mice, failed to show any DTH response. But ten days after challenge the mice which were immunized with Fr.1 and Fr. 2 (s.c.) showed significant DTH response as demonstrated in Fig. 1. Although 60 days after challenge the DTH responses had been suppressed in all groups, but still s.c. immunized mice with Fr.1 and Fr.2 were shown higher DTH values than other groups ($P<0.05$) (data was not shown).

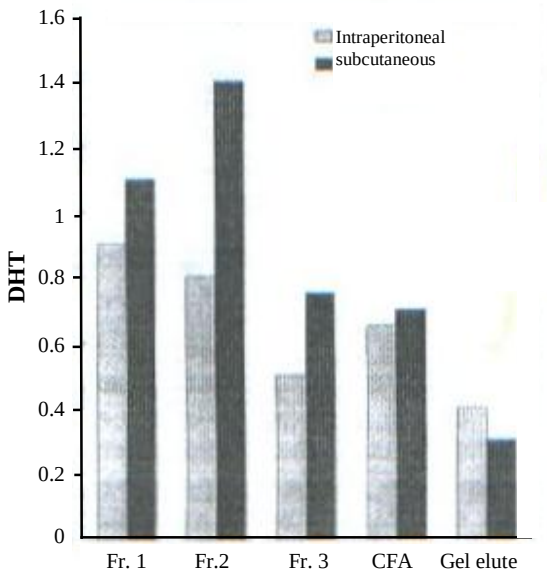

Fig. 1. DTH values 24 hours after skin testing with FT leishmanial antigen in the footpad of immunized and control BALB/c mice (n = 6), 10 days after infection with infective *L. major* promastigotes. The immunization protocol was consisted of 3 injections (by intraperitoneal or subcutaneous route) of a proteinic fraction of *L. major* promastigotes. First injection was mixed with CFA adjuvant and the two others without it within a 10 days interval. The control mice were received one injection (i.p., s.c.) either eluate of polyacrylamide gels which had not been loaded with *leishmania* promastigotes or CFA alone.

Lymphocyte proliferation response. The proliferation response of lymph node cells of all mice to FT antigen was studied by lymphocyte proliferation assay (LTT) and the results were shown by means of stimulation index (SI), (Fig. 2). In spite of the lack of DTH in unchallenged immunized mice, lymph node cells from these animals responded to FT leishmanial antigen in LTT assay. As shown in Fig. 2, mice immunized with Fr.1 and Fr. 2 (s.c. and i.p.) showed significantly higher responses (SI) ($P<0.05$) than mice immunized with Fr.3 and control groups.

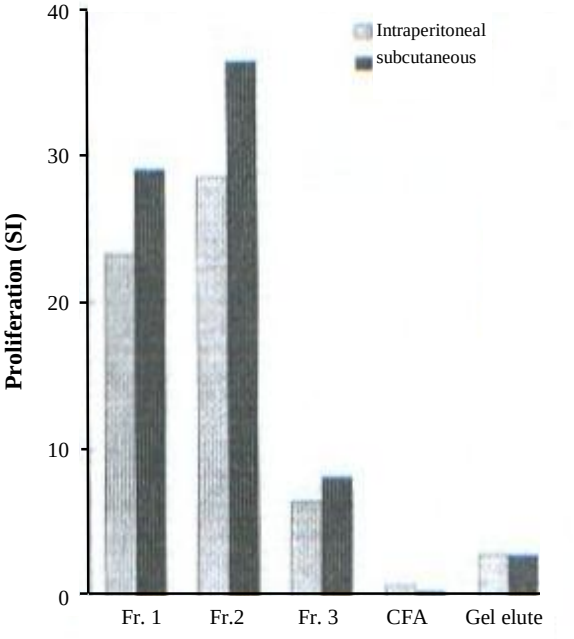

Fig. 2. Proliferation of lymph node cells of immunized and control BALB/c mice (n=3) against FT leishmanial antigen, 2 weeks after the last immunization. Proliferation was measured with [^{3}H] thymidine incorporation , and is shown as stimulation index.

Challenging with virulent parasites. As shown in Fig. 3, 12 weeks after challenging of mice with virulent *L. major* parasites, their lesion size was measured within the control and the immunized groups. The control mice and mice which were immunized i.p. by any of the three fractions developed a progressive infection at the injection site. Meanwhile the mice which were immunized s.c. with Fr.2 resulted in partial protection with a delay in the onset of lesions (Fig.4). Also s.c. injection of Fr. 1 and CFA alone, caused a delay in the onset of lesions and smaller lesions, 12 weeks after the challenge (Fig. 3).

DISCUSSION

In an attempt to better understanding the protective effect of *L. major* antigens in murine model, three fractions of *L. major* promastigotes were isolated by SDS-PAGE and electro-elution. These fractions were used for immunization of BALB/c mice by s.c. or i.p. routes. Subsequently the mice

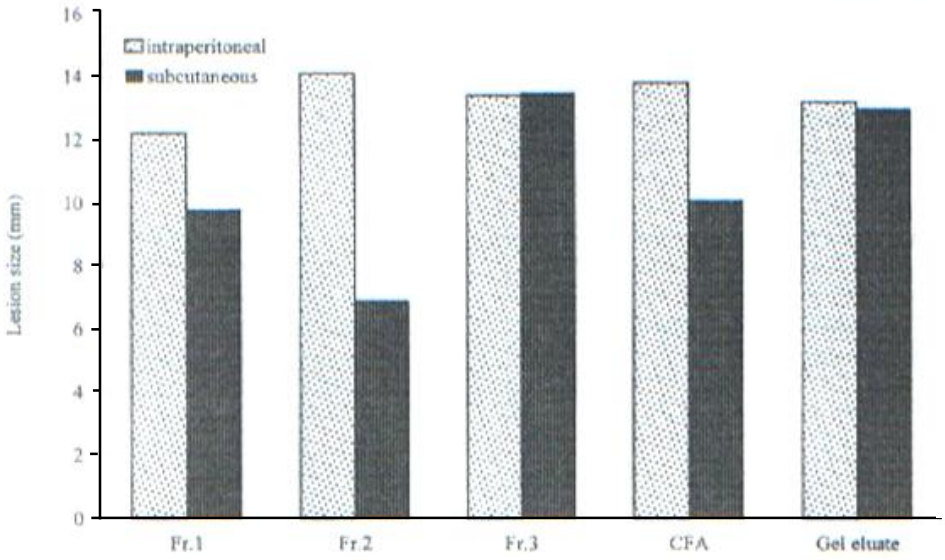

Fig. 3. The lesion size of immunized and control BALB/c mice (n = 10), 12 weeks after challenging with $2x10^6$ *L. major* promastigotes at the base tail.

were challenged with virulent parasites. For studying the protective effects of the obtained fractions, the development of lesion size in injection site in immunized and control mice were compared. Also the proliferative response of their lymphocytes against leishmanial antigen was evaluated *in vitro.* The DTH skin test responses were investigated in these animals. The obtained results showed that the subcutaneous immunization with Fr.2 and in some extent with Fr.1 can induce partial resistance against leishmanial infection. Fr.1 and Fr.2 respectively comprised of proteins with 40-60 and 60-80 kDa molecular weights. These results are in agreement with other findings in other species of *Leishmania,* which has used a 64-97 kDa fraction of *L. infantum* mixed with muramyl dipeptide (MDP) as an adjuvant for immunoprophylactic properties in BALB/c mice against leishmaniasis [6]. The s.c. immunized mice with Fr.1 had smaller lesion sizes than mice which were immunized with Fr.3 and control mice. We presume that, there may also present some protective antigens in Fr.1 as well as Fr.2, as suggested by McMahon-Pratt and Hand-man; the surface antigen gp46/M2 and the related parasite surface antigen 2 (PSA) has induced significant protection in animal models when delivered with proper adjuvant [9, 10].

The lesion size in control mice which had received CFA alone was significantly smaller than the other control mice ($p<0.05$). CFA is a potent adjuvant which may stimulate non specifically the cellular immunity of host against *L. major* infection, and so it can be used as a potentiator of immune system, and nonspecific activator of macrophage [11, 12]. It appears that there might be some common cross-reactive antigens between *Leishmania* and *Mycobacterium* spp., which can stimulate the immune responses against *Leishmania* infection, as suggested by Kalipada Kar [13].

Ten days after challenging, the DTH reaction was positive, and remained positive for 60 days. Therefore, DTH positive responses may be considered as a tool to differentiate protective (Thl) and disease promoting (Th2) subset of $CD4^+$ T cells [14-16], Thl cells mediated a DTH reaction when mice were injected by specific antigen to the footpad [17], while Th2 cells cannot.

Our data showed that immunization with three fractions of *L. major* parasites resulted in stimulation of cell mediated immune response (LTT), while fraction 2 (s.c.) showed partial protective properties (smaller lesion size) and the others failed to induce any protective immunity against leishmanial infection. Although T-cell response alone may not predict which antigens are protective [18]. In general, these observations indicated that

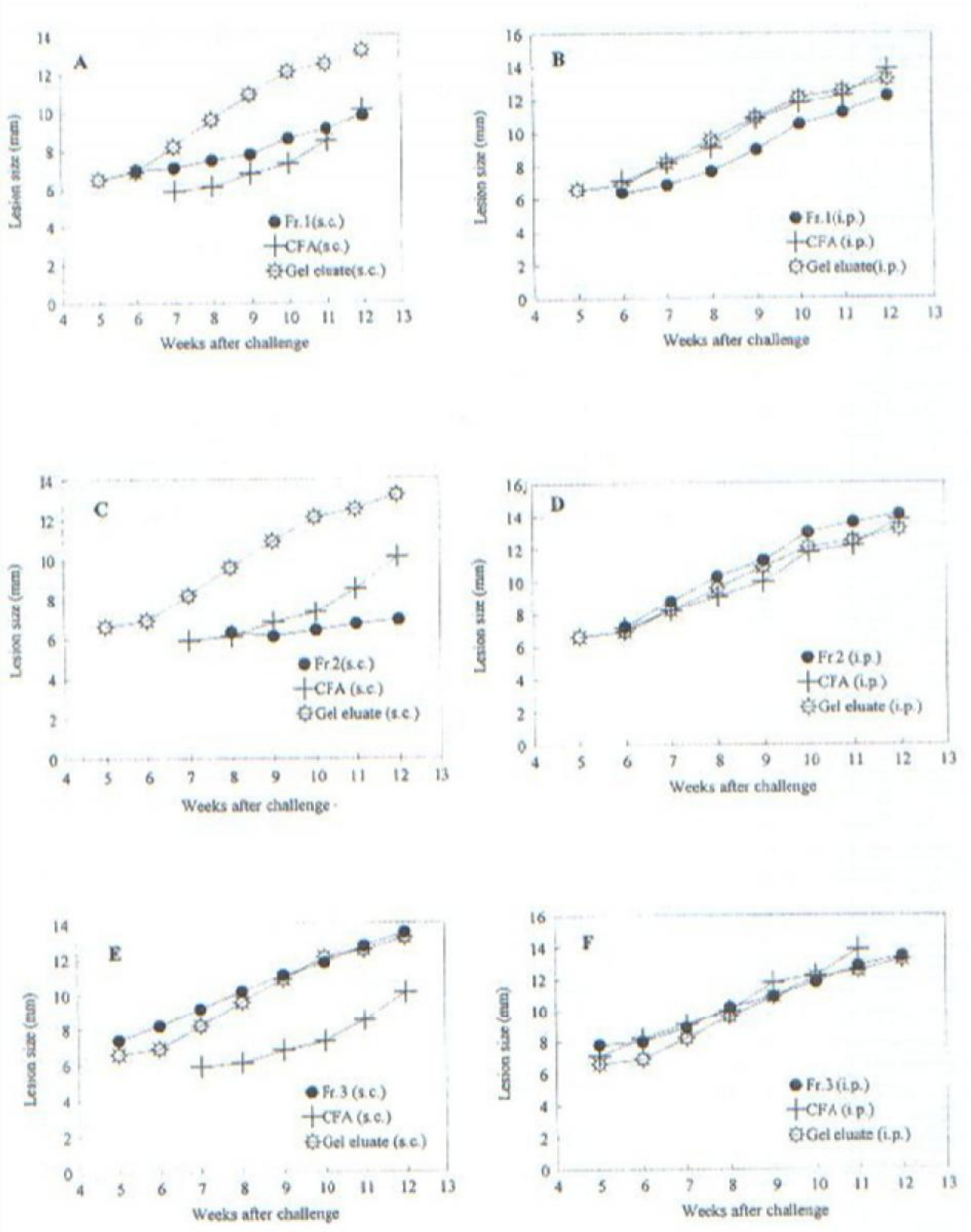

Fig. 4. Comparative lesion development at the base tail of immunized and control BALB/c mice (n=10), during 12 weeks after challenging with 2×10^6 infective *L. major* promastigotes

identification of antigens recognized by T-cells, is an important primary step for defining a protective immunogen, and empirical immunization studies in vaccine models are crucial in defining a leishmanial vaccine.

In each immunization protocol many factors such as the amount of immunogen , route of administration, type of adjuvant are involved and are very important in induction of appropriate T cell subsets [14]. In summary, we have found that a 60-80 kDa fraction of *Leishmania major* when administered subcutaneously mixed with CFA could partially protect BALB/c mice against leishmanial infection. More investigation is needed to define the protective immunogens presented in fractions 1 and 2, and biochemical studies of these immunogens are necessary.

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