Immunization against *Leishmania donovani*: Efficacy of *Mycobacterium habana* in combination with killed promastigotes in hamsters

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The efficacy of killed *Leishmania donovani* promastigotes in combination with *Mycobacterium habana* was assessed against *L. donovani* infection in golden hamsters. The prophylactic potential of the combined immunogens following challenge by *L. donovani* amastigotes was substantially enhanced (65.5%) as against any immunogen given singly. The specific humoral response to leishmania promastigote antigen, as assessed by enzyme-linked immunosorbent assay (ELISA), revealed elevated antileishmanial antibodies on day 20 post challenge (p.c.) in the unvaccinated challenged controls and the vaccinated groups receiving either formalin-killed promastigotes (FKP) or *M. habana*. However, the level of antibodies was subdued in the group receiving combined immunization with FKP and *M. habana*. The cellular immune response (CMI) was studied by both *in vivo* (delayed type of hypersensitivity, DTH) and *in vitro* by macrophage migration index (MMI). There was substantial increase in DTH response to both sensitizing antigens, i.e. FKP and habanin, in animals immunized with combined immunogens (FKP + *M. habana*). The MMI response in the animals receiving FKP + *M. habana* was 0.71 compared to unvaccinated challenged animals (0.43). The immunization with *L. donovani* in combination with *M. habana* appears to hold promise in the containment of leishmania and needs further exploration.

VISCERAL leishmaniasis (VL) is a chronic vexed problem of the tropical world afflicting about 15 million people across the globe with heavy morbidity and considerable mortality[1]. Vaccines are the ultimate, universally accepted preventive measures against several infectious diseases. The usage of vaccines avoid the problems of drug resistance and they are potentially safer, cheaper and more efficacious for the containment of diseases in the community[2].

Convit *et al.*[3] have achieved significant protection against human cutaneous leishmaniasis following immunization with live BCG along with heat-killed *Leishmania mexicana* promastigotes. But this immunogen (BCG) has not been recommended as a mass immunizing agent for various reasons[4,5]. Leishmaniasis and leprosy having several common facets in pathogenesis and the progression of disease could be cross linked for devising common strategy for their control. Since BCG has many drawbacks, the other immunogenic and nonpathogenic strain of *Mycobacterium* – *M. habana* (*M. simiae* Serovar-1), which is highly effective against *M. tuberculosis*, *M. leprae* and *M. ulcerans* infections[6–9] could also be beneficially explored as an alternative agent for immunization against VL.

Encouraged with the findings of Convit *et al.*[3,10], the prophylactic potential of this organism (*M. habana*) was assessed in combination with formalin-killed promastigotes (FKP) against *L. donovani* challenge in hamsters.

Laboratory-bred male golden hamsters (*Mesocricetus auratus*, 45–50 g) from the Institute's animal house facility were used as the experimental hosts. The animals received standard rodent pellet (Lipton India Ltd.) and water *ad-libitum*, and were maintained in regulated light and temperature conditioned rooms.

The WHO reference strain of *L. donovani* (MAN/IN/80/Dd8) was obtained in 1979 from Imperial College, London. The strain since then has been maintained in this laboratory through serial passage (amastigote to amastigote) in hamsters.

The formalin-killed promastigotes were used for the purpose of immunizing the hamsters and to evaluate delayed type of hypersensitivity (DTH) response in them. The viable promastigotes were harvested from L-15 medium, washed thrice in PBS and killed by suspending in 0.1% formalin for 30 min at 37°C and kept at 4°C overnight[11]. These formalin-killed promastigotes were washed thrice by centrifugation with PBS to remove excess of formalin, counted in a haemocytometer, resuspended in PBS at $1 \times 10^7/0.1$ ml concentration and stored at 4°C until use. In order to ensure that formalin treatment killed all parasites, an aliquot from formalin-treated suspension was inoculated into culture medium, incubated at 26°C for 7–10 days and later examined microscopically for viability.

This antigen was used in ELISA. The promastigotes were cultivated in L-15 medium (Gibco, USA), supplemented with 10% tryptose phosphate broth (Hi Media, Bombay, India), 0.1% gentamycin (Biovaccines Private Ltd., Chevella, India) and 10% foetal calf serum (Biological Industries, Halmelz, Israel)[12]. The parasites were harvested by centrifugation at 2000 rpm for 15 min, washed three times with phosphate-buffered saline (PBS) and stored at −20°C until needed. Soluble somatic antigen was prepared according to the method of Choudhury *et al.*[13] After assessing protein contents[14], the antigen was distributed in small aliquotes and stored at −70°C until use.

Mycobacterium habana TMC 5135 (now *M. simiae* Serovar-1) having three strong markers – the photochromogenicity, strong niacin positivity and catalase

activity, was obtained from Trudeau, Mycobacterial Culture Collection Centre, New York. It was maintained on Lowenstein–Jensen (L–J) medium. The organisms grown in liquid Sauton's medium from two weeks upto the logarithmic phase were harvested and washed thrice (by centrifugation) with saline at 4°C. These were finally suspended at a concentration of 15 mg/ml (wet weight). The suspension was inactivated to sterility with 30 megarads of gamma irradiation from 60 cobalt source at 300 K rads. Sterility of the irradiated vaccine was confirmed by absence of any growth upon inoculation on L–J medium and nutrient agar slants and were stored in suitable aliquotes at –20°C until use.

The experiment involved four groups of hamsters. Three groups were immunized intradermally either with FKP (1×10^7/animal) alone, M. habana (0.75 mg/animal) alone or the two in combination, on day 'O'. The fourth unimmunized group served as control. All the animals were challenged intracardially with 1×10^7 amastigotes on day 21 of immunization. The prophylactic efficacy of immunogens was assessed, following autopsy, on day 20 post challenge (p.c.) by counting the number of amastigotes 500 cell nuclei[-1] in splenic dab smears. Blood was collected to extract serum. Macrophages from peritoneal cavity were isolated for immunological tests.

Enzyme linked immunosorbent assay (ELISA) was carried out as described by Voller et al.[15]. Sera from all the four groups were collected on day 20 p.c. ELISA plates (Nunc) were coated with soluble promastigote antigen ($1\,\mu g$ in $100\,\mu l$ well[-1])[16]. PBS at pH 7.2 and containing 0.05% Tween-20 served as the diluent. Goat antihamster IgG (H + L) labelled with peroxidase (Kirkegaard and Perry Laboratories Inc., Gaithersburg, USA), in a dilution of 1 : 1000 was used as conjugate and the assay was read (in a series 700 microplate reader, Cambridge Technology) at 492 nm.

Hamsters from each immunized and control group, were tested for DTH response on day 21 post immunization (before challenge) and on day 20 p.c. The two antigens, FKP and habanin (1×10^7 and $100\,\mu g$ protein respectively in 0.1 ml hamster[-1]) were injected intradermally in the right foot pad. Left foot pad served as control and injected with similar amount of normal saline. At 24, 48 and 72 h of antigen injection, the induration was measured with the help of a dial caliper (Schnelltaster, Germany). The degree of hypersensitivity was expressed as increase of foot pad thickness over the control values[17].

Macrophage migration index (MMI), a correlate of cell-mediated immunity and macrophage activation, was carried out on day 20 p.c.[18].

The ratio of the area of migration of macrophages from immunized challenged and unimmunized challenged groups to that of the cells from normal control was designated as the MMI.

$$MMI = \frac{W_1}{W_2},$$

where W_1 = macrophage migration area from immunized challenged and unimmunized challenged animals. W_2 = macrophage migration area of normal control animals.

Student's t test was applied for the statistical analysis of the data.

Formalin-killed promastigotes failed to elicit any protection whereas immunization with M. habana yielded 39.2% inhibition of parasite multiplication. M. habana when combined with FKP (which is otherwise ineffective), the parasitic inhibition was substantially enhanced to 65.56% ($P < 0.01$), (Table 1).

The antibody titre at the serum dilution of 1 : 100 has been presented in Figure 1. In the infected controls (unimmunized challenged) there was sharp increase in the antileishmanial antibodies by day 20 p.c. (0.826). Similarly, the two immunized groups receiving either FKP or M. habana have high antibody levels (0.784 and 0.692 respectively). However, the rise in antibodies was subdued in the group immunized with combined immunogens (0.508).

Unimmunized challenged animals elicited no DTH

Table 1. Prophylactic effect of different immunogens against L. donovani infection in hamsters

Groups	Intradermal immunization	Dose/hamster (in 0.1 ml)	No. of animals immunized	Parasite burden Mean ± SD	% Inhibition Mean ± SD
I	Experimental control	0.1 ml of normal saline	15	48.3 ± 8.36	–
II	Formalin-killed promastigotes of L. donovani (FKP)	1×10^7	15	46.25 ± 8.73	4.24 ± 3.08
III	M. habana	0.75 mg weight (3.13×10^8 cells)	15	29.36 ± 4.47	39.21 ± 6.19
IV	FKP + M. habana	1×10^7 + 0.75 mg	15	16.63 ± 3.63	65.56 ± 4.26**

*, Amastigotes/500 splenic macrophages.
**, Significant ($P < 0.01$).

response (48 h post sensitization) to any of the sensitizing antigens i.e. FKP and habanin as evident in Figure 2. Similarly, the animals immunized either with FKP or *M. habana*, responded weakly to homologous or heterologous antigens (5.66–16.66% increase in foot pad swelling). However, significant DTH response was observed in hamsters receiving combined immunogens (FKP + *M. habana*) with FKP and habanin, and measured footpad swelling was 25.67% and 37.50% respectively on day 20 p.c.

Figure 3 depicts the data on MMI of different categories of hamsters. The migration of macrophages from unimmunized challenged hamsters on day 20 p.c. was very small (0.43) and was comparable to the animals immunized with FKP (0.46) and *M. habana* (0.54) alone. However, the animals receiving combined immunogens (FKP + *M. habana*) MMI record was 0.71.

The available therapeutic agents to combat VL are inadequate[19,20] and the alternate measures, viz. prophylaxis and immunotherapy are yet to emerge. Since vaccines have discernible advantages over therapy[21], we

ventured to search for effective immunogen(s) to induce immunity against VL.

Despite many favourable reports, the action of BCG against tuberculosis, leprosy and leishmaniasis[4,5,22] is unpredictable in different geographical regions. *M. habana* grows luxuriously in cultures and is nonpathogenic to several species of animals (Singh *et al.*, unpublished data). This organism affords significant protection against tuberculosis and leprosy[7,9]. Since leprosy and leishmaniasis are characterized by severe impairment of CMI and *M. habana* is protective against leprosy, it is likely that the antigenic cross-reactivity may exist between species of the genera *Mycobacterium* and *Leishmania*[23–25].

In the present study this combination has worked effectively in reducing the challenge infection to a considerable extent (65.5%). This concomitant immunity to leishmania might be either due to the adjunctive action of common antigens or the adjuvant effect of *M. habana*. Holbrook *et al.*[11] and Holbrook and Cook[26] have also reported that injections of killed promastigotes alone conferred no measurable resistance against leishmania infection but significant protection was observed when combined with glucan which had an adjuvant effect.

As in clinical leishmaniasis, the increased antibody level in hamsters corresponded with the level of infection. The animals immunized with FKP combined with *M. habana*, showed low profile of antibodies (0.508), as they had low grade of infection i.e. 34.4%, in comparison to unimmunized challenged animals (0.826).

It has been reported that in leishmaniasis the number of circulating T-lymphocytes severely decline due to the stimulation of Th2 cells which secrete IL-4 and IL-10 and exert inhibitory effect on the disease protective cells (Th1)[27]. However, this factor may not interfere with the generation and activity of cells that cooperate with B lymphocytes, as such, the B cells activity remains undisturbed leading to the production of high level of antibodies. This is a common feature in all kala-azar cases but the protective role of these antibodies has not
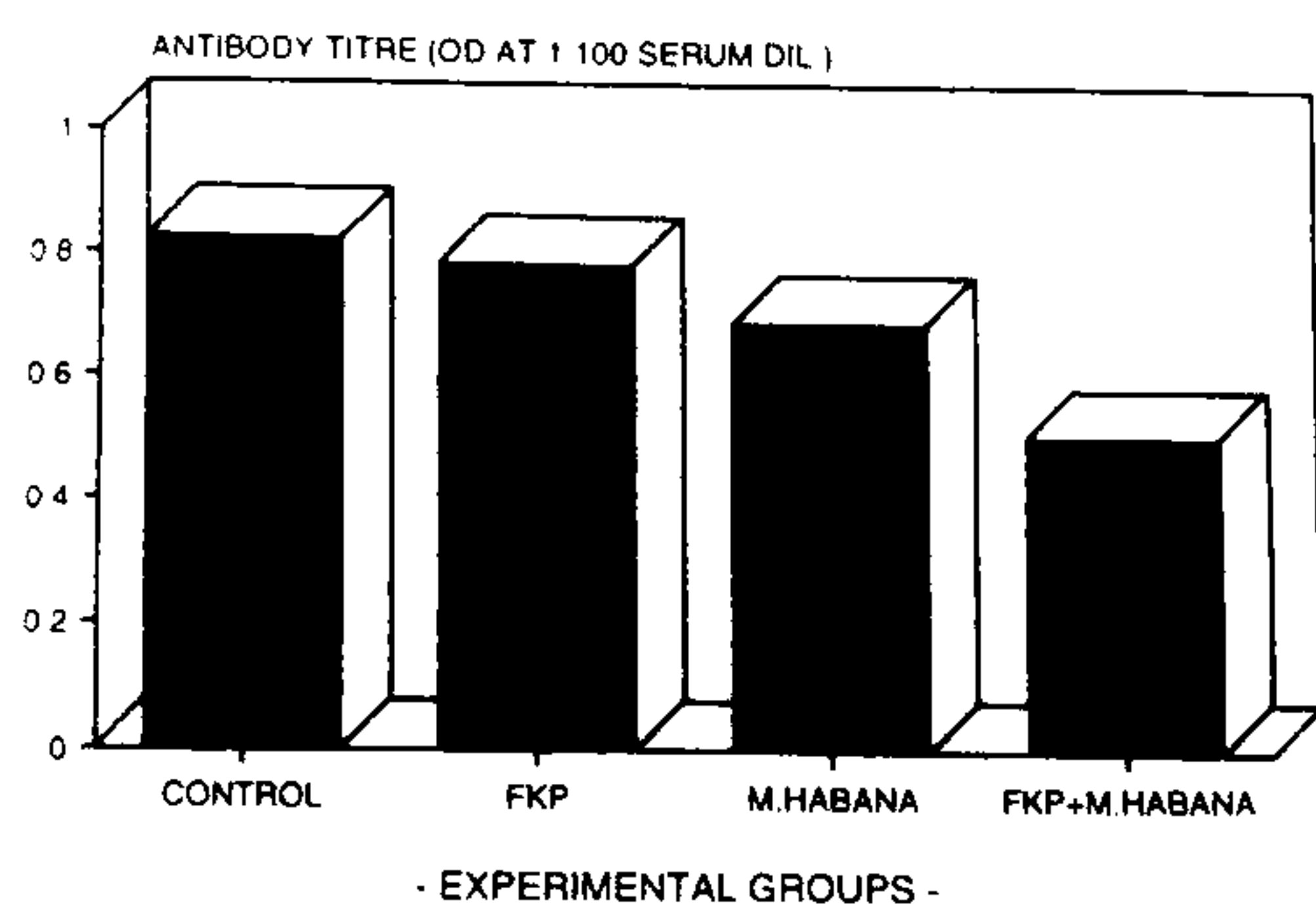

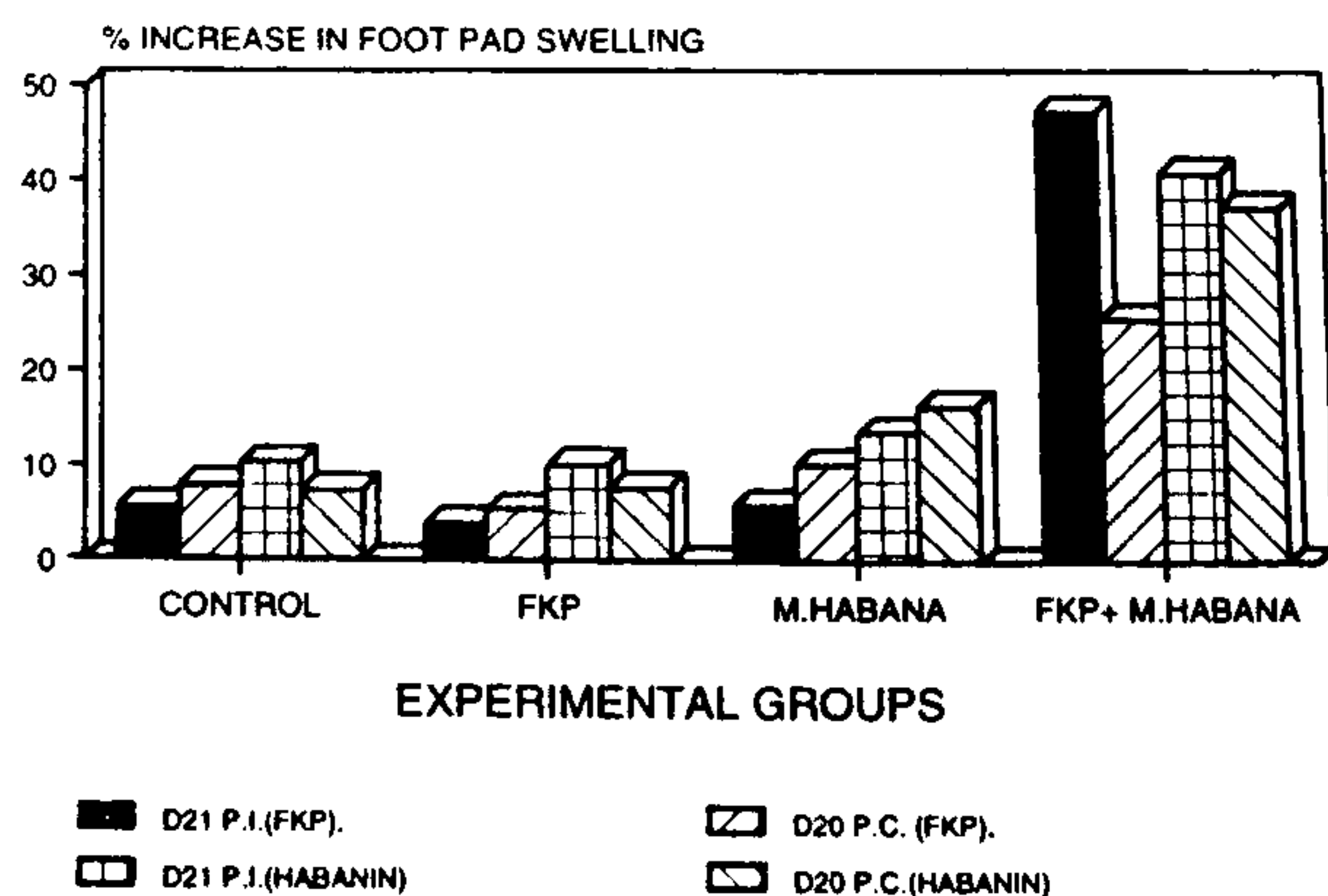**Figure 1.** Antibody profile of immunized hamsters on day 20 post challenge.

Figure 2. DTH response of immunized hamsters (48 h post sensitization).
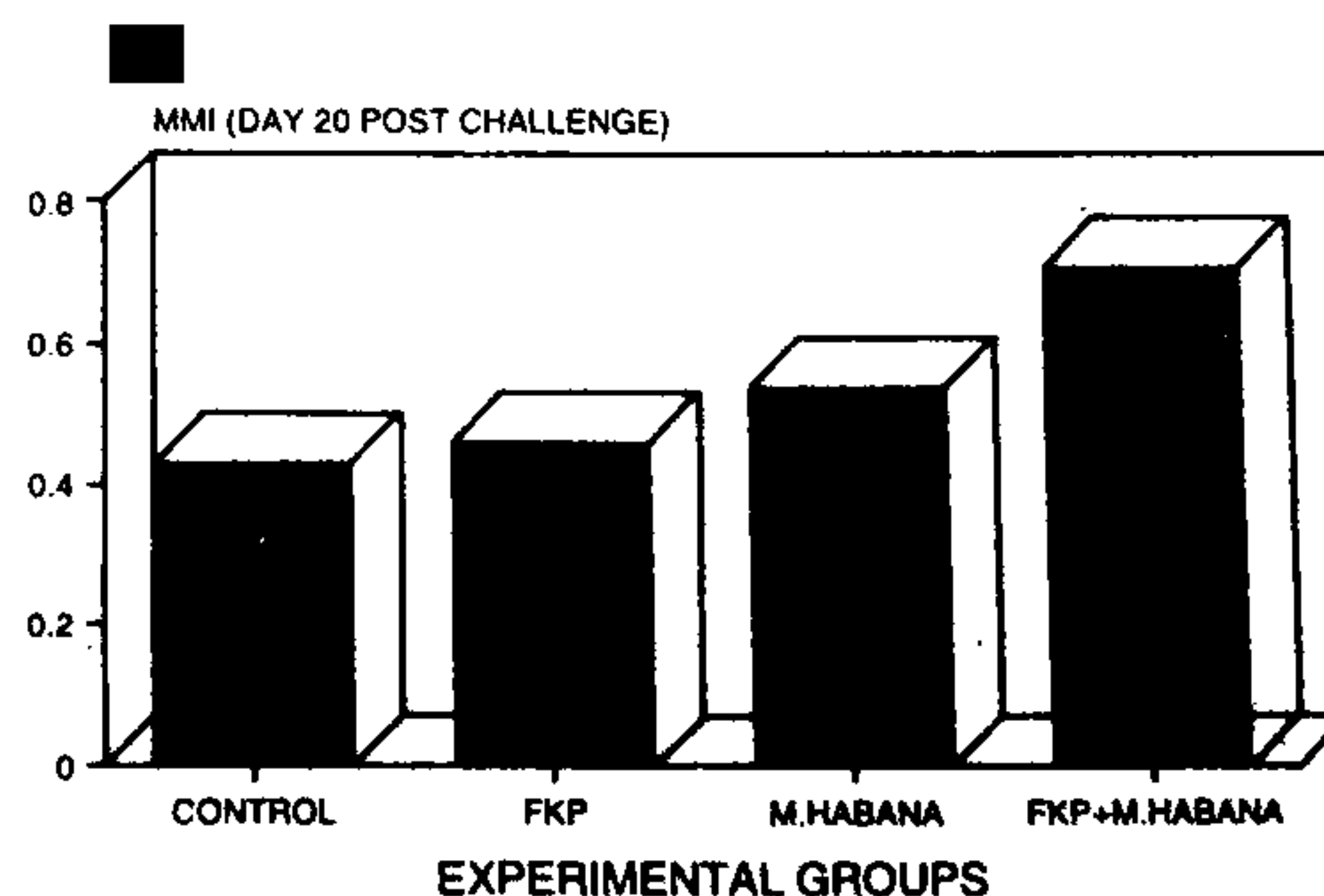

Figure 3. Macrophage migration index of immunized hamsters on day 20 post challenge.

been established till date. In the present observation almost similar pattern was noticed. The phenomenon appears to have more relevance in predicting the degree of infection.

Macrophage activation is the indicator of nonspecific response against the foreign invaders. To measure the degree of activation, macrophage migration is a relative parameter. The unimmunized challenged hamsters showed declining trend of migration index with the advancement of infection. Immunization with single antigen, either with FKP or with *M. habana*, exhibited decrease in MMI after the challenge though the inhibition was not significant. On the other hand, there was only slight suppression in macrophage migration index in hamsters immunized with combined immunogens and was comparable to normal control. The DTH response was also found increased in animals immunized with combined immunogens. These findings corroborate with earlier reports[12,28].

The preliminary studies on cross protection by *M. habana* have opened up a new vista in the vaccination against experimental leishmaniasis where antigenic advantage of nonpathogenic organism could be beneficially exploited to produce functional immunity against a fatal disease, leishmaniasis, and call for more organized and concerted efforts to investigate the potentiality of *M. habana* in VL.

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Anomalous fluoride in groundwater from western part of Sirohi district, Rajasthan and its crippling effects on human health

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Anomalously high concentration of fluoride (upto 16 ppm) has been observed in dug/tube well water, which is being used for drinking and irrigation purposes, around Palri, Andor and Wan villages, in western part of Sirohi district, Rajasthan. Fluoride concentration in groundwater is much higher than the permissible limit of 0.6–1.5 ppm of fluoride recommended for potable purposes. Water samples with more than 5 ppm fluoride are confined to Andor and Wan villages. Mottling is commonly observed in people of this area with a few cases of crippling

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