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Adjuvant Effect of Leishmania major Promastigotes on the Immune Response of Mice to Ovalbumin

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ABSTRACT

The immune responses of mice immunized with ovalbumin (OVA) together with killed L. major (KLM) promastigotes as adjuvant were studied. Three doses (5×10^7, 1×10^8 and 2×10^8) of KLM combined with OVA (100 «g) were injected into the groups of C57BL/6 mice. BCG and complete Freund's adjuvant (CFA) were used as control adjuvants. Lymphocyte proliferation and antibody titers were determined, and IFN-¥ and IL-4 were measured in the supernatants of lymph node cell cultures. Results showed that immunization using OVA mixed with KLM enhanced the in vitro proliferative response of T-cells to the antigen and resulted in the production of increased levels of IFN-¥ (2800-3700 pg/ml) relative to the mice injected with OVA alone (1750 pg/ml). In the mice receiving OVA + 5×10^7 KLM, the production of IL-4 remained lower (18, 20 pg/ml) than OVA alone (105, 109 pg/ml) and almost was similar to that of observed in mice inoculated with OVA + BCG, leading to high IFN-¥/IL-4 ratios. Using higher doses of KLM (1×10^8), the IL-4 responses were of the same magnitude as or higher than the responses of mice inoculated with OVA + CFA. Antibody titers to OVA were also strongly boosted at the highest KLM dose. These findings indicate that KLM may function as an adjuvant, and its dose plays a role in the eventual outcome of the response. Inoculation of the mice with a low dose of KLM (5×10^7) tends to promote a Th1-type response. Iran. Biomed. J. 6 (4): 123-128, 2002

Keywords: *L. major*, Adjuvant, Ovalbumin, Immune response, Mice

INTRODUCTION

Leishmania parasites are protozoan micro-organisms that infect the cells of the mononuclear phagocyte series in their vertebrate host. The infection elicits a strong humoral or cell-mediated immune response, or both, depending on the infecting species and on characteristics of the host. A number of similarities can be noted between the immune responses elicited by these organisms and Mycobacteria: a) Protective immunity induced by both organisms is T-cell mediated and macrophage plays a crucial role as immune effector cell [1-3]; b) Leprosy and leishmaniasis are characterized by well-defined polar forms in both infections. The localized tuberculoid form is accompanied by strong lymphocyte proliferation and the IFN-γ production in response to antigen challenge in vitro [4]; c) Polarization of the immune responses towards protection (induced by the Th1 subset) or to exacerbation (induced by the Th2 subset) has been documented in both L. major and M. leprae infections [5]; d) In experimental murine leishmaniasis, innate immunity is controlled by Lsh gene that is thought to be identical to the Bcg gene [6]; e) T cell clones from individuals vaccinated against BCG or killed Leishmania show significant INF-γ production. Both types of clones are of $CD4^+$ phenotype, and the antigen recognized by these clones may be involved in protection against re-infection [7, 8]; f) the existence of antigenic cross-reactivity between both organisms has been well documented [9, 10].

The use of BCG as an adjuvant has been well established for many years [11]. Immunotherapy of the patients with leishmaniasis using killed parasites plus live BCG has been carried out successfully [12]. Furthermore, in vaccine trials using killed L. major candidate vaccine, BCG is commonly used as

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an adjuvant [13-16]. However, adverse effects or complications have been reported in the use of BCG, including inflammatory arthritis and autoimmune reactions [17-19]. On the other hand, inoculation of killed Leishmania is fully innocuous, as proved by the use of leishmanin as skin test antigen (Montenegro test) for several decades [20]. Leishmania promastigotes are easily cultured in monophasic media. It was therefore of interest to examine whether L. major could exert an adjuvant effect on the immune response of mice to some unrelated antigens.

In the present work, the potential adjuvant effect of KLM promastigotes was tested in mice. Ovalbumin (OVA), a T-dependent antigen, also used as a carrier in experimental model [21], was chosen as an immunogen, and CFA or BCG as control adjuvants. Antibody responses, T-cell proliferation and cytokine production were assessed.

MATERIALS AND METHODS

Animals and parasites. Female C57BL/6 mice (7-8 week-old) were used in these experiments and the original stock was purchased from IFFA CREDO (St. Gerrmain-sur-l'Arbresle, France). The experimental animals were obtained from the Animal Breeding Center of the Pasteur Institute of Iran. The strain of *L. major* (MRHO/IR/75/ER), kindly provided by Dr. E. Javadian (School of Public Health, Tehran University of Medical Sciences, Iran) was used in the experiment as adjuvant. Parasites were grown in Novy-MacNeal-Nicole (NNN) medium, and then were transferred to RPMI (Sigma, Germany) supplemented with 0.2 mM L-glutamine, 100 U/ml penicillin, 100 μg streptomycin and 15% fetal bovine serum (FBS). The stationary phase promastigotes were harvested, washed, killed by 0.05 % thimerosal, disrupted by 7 cycles of freezing and thawing and then kept at -70°C until use.

Immunization protocol. Seven groups of 10 mice each were injected with OVA alone (100 μg/100 μl), OVA + 5×10^7 KLM, OVA + 1×10^8 KLM, OVA + 2×10^8 KLM, OVA + BCG (Pasteur Institute of Iran, 6-7 × 10^7 CFU/ml), and OVA + CFA (Sigma, 50 μl), respectively, and a control group injected only with PBS. Each mouse in group 2-6 received OVA in 100 μg/50 μl + adjuvant in 50 μl volume. A total volume of approximately 100 μl was injected subcutaneously at the base of the tail. The amount of OVA used for each mouse was 100 μg and the same amount was injected to 5 mice as a booster without adjuvant one week later.

Lymphocyte proliferation assay. Inguinal and periaortic lymph nodes (LN) of mice were removed 20 days after the second immunization. Pooled LN cells from 5 mice were cultured as described [22]. Briefly, the viability of the cells was evaluated with trypan blue exclusion, and viable lymphocytes (4×10^5) were cultured for 4 days in 200 μl enriched RPMI 1640 medium (supplemented with 2 mM L-glutamine (Sigma), and 10% FBS, 10 mM HEPES, 5×10^{-5} M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin) in the presence of 250 μg/ml of OVA, a concentration found in pilot experiments to provide an optimal response. Con A was used as a positive, and medium alone as a negative control. The proliferative response to antigen was quantified by measurement of ^{3}H-methyl-thymidine uptake (Amersham, UK). This test was repeated three times.

Cytokine production and assessment. Mono-nuclear lymph node cells (2×10^6) were cultured as above in 1.0 ml complete medium in the presence of 250 μg/ml OVA in flat-bottomed 24-well micro-plates for four days. Supernatants were harvested and stored at -70°C, and the concentrations of IFN-γ and IL-4 were quantified by a sandwich ELISA technique using commercial kits (Genzyme, USA) according to the procedure of the manufacturer. Samples were tested in duplicates. Data were calculated from reference curves obtained using standards provided in the kits.

Antibody measurement. ELISA was used for antibody measurement as described [23]. Briefly, 96-well microplates were coated with ovalbumin (150 μg/well) in carbonate buffer (pH 9.6) and incubated overnight at 4°C. Serum samples were diluted in duplicates in total volume of 200 μl PBS at 37°C for 1 h, followed by a peroxidase conjugated anti-mouse IgG and IgM (Sigma), and substrate (o-Phenylenediamine in 10 ml citrate buffer (pH 5.0), plus 0.02% H_2O_2. The plates were read at 490 nm.

RESULTS

Induction of a proliferative response. Pre-liminary experiments indicated that the injection of

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OVA plus a low dose of KLM (4×10^7) capable of increasing the proliferative response in LN cells culture against OVA antigen when compared to mice injected with OVA alone (data not shown). In further experiments, mice were immunized with OVA + higher doses of killed parasites (5×10^7, 1×10^8 or 2×10^8), which resulted in a much higher proliferative responses of LN cells to ovalbumin. The results obtained using a dose of 10^8 promastigotes were comparable to those achieved in mice injected with OVA + BCG, especially after the first immunization. As shown in Figure 1, KLM increased the proliferative response to ovalbumin about 12-folds (first immunization), and about 3-folds (second immunization), relative to OVA alone. Using student's *t*-test a significant difference was observed in first ($p<0.001$) and second ($p = 0.001$) immunization. Similar results were obtained in comparison of mice injected with OVA + BCG and mice injected with OVA alone ($p<0.001$ for both first and second immunization). Interestingly, after the first immunization, the proliferative response of LN cells of mice inoculated with OVA + CFA was significantly ($p = 0.002$) lower than that of cells from mice receiving OVA + KLM.

Cytokine production. Immunization of mice with OVA + KLM enhanced IFN-γ production in OVA-stimulated LN cell cultures relative to the lymph node cells from the mice injected with OVA alone. As shown in Table 1, the effect of KLM was similar to that of BCG after both the primary and the secondary immunization. However, in mice immunized with OVA + CFA, production of IFN-γ remained low after the first immunization and increased after the second one.

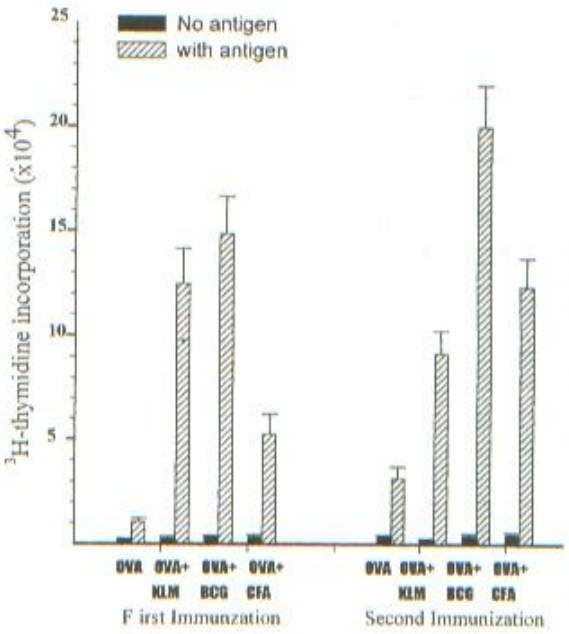

Fig. 1. Proliferative responses of lymph node cells from the mice immunized with ovalbumin (OVA): adjuvant effect of KLM. Mice (10 per group) were immunized at the base of the tail with OVA + 10^8 killed L. major (KLM), OVA alone, OVA + BCG or OVA + CFA. Five mice from each group were boosted after one week by inoculation of OVA alone. Twenty days later, periaortic and inguinal lymph nodes were harvested and lymphocytes were cultured for 4 days in the absence or presence of OVA (250 μg/ml). Proliferation was assessed by measurements of ^{3}H-TdR uptakes during the last 18 h of culture. Each column represents the arithmetic mean of triplicate determinations and vertical bars show the limits of one standard deviation. The results shown are representative of three independent experiments.

Table 1. Balance between Th1 and Th2 cytokine production in lymph node cell cultures derived from mice immunized with ovalbumin and different adjuvants.

Adjuvant	No. of injections	Cytokine Concentration (pg/ml)		IFN-¥/IL-4
		IFN-¥	IL-4	
No adjuvant	1	1,750	105	17
	2	1,750	109	16
KLM 5×10^7	1	3,000	20	150
	2	3,000	18	167
KLM 10^8	1	3,700	120	31
	2	2,820	131	22
KLM 2×10^8	1	2,800	70	40
	2	2,900	52	56
BCG	1	3,100	35	89
	2	3,000	62	48
CFA	1	780	73	11
	2	2,750	118	23

Mice received one or two injections of 100 μg of OVA. Adjuvant was added to the first inoculum. The inguinal and periaortic lymph node cells from 5 mice per group were pooled and re-stimulated *in vitro* with OVA (250 μg/ml) for 4 days, prior to assessment of cytokine concentrations in the culture supernatants.

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The production of IL-4 in OVA-stimulated LN cells of the mice immunized with OVA plus the smaller dose of KLM (5×10^7) was markedly lower when compared to the mice immunized with OVA alone, after both the primary and the secondary immunizations. These responses were similar to those of control mice receiving OVA + BCG, particularly after the first immunization. Likewise, the ratio of IFN-¥/IL-4 was distinctly higher in the group of mice injected with OVA plus the lower dose of KLM (5×10^7), relative to all the other groups. In contrast, higher and somewhat variable IL-4 levels were observed in cultures of OVA-stimulated LN cells of the mice immunized with OVA plus higher doses of KLM, correlating with the results of the mice injected with OVA + CFA (Table 1).

Antibody responses. No obvious effect of KLM on the primary antibody response to OVA could be observed. However, high titers of anti-OVA IgG antibody were obtained in mice immunized with OVA + 2×10^8 KLM, then boosted with OVA alone, as shown in Figure 2. Lower doses of KLM (5×10^7) were ineffective as adjuvant for antibody production. Higher antibody levels still were observed in the mice injected with OVA + CFA, whereas mice receiving BCG as adjuvant developed only modest titers.

measured by stimulation of the proliferative response of lymph node lymphocytes *in vitro*, and

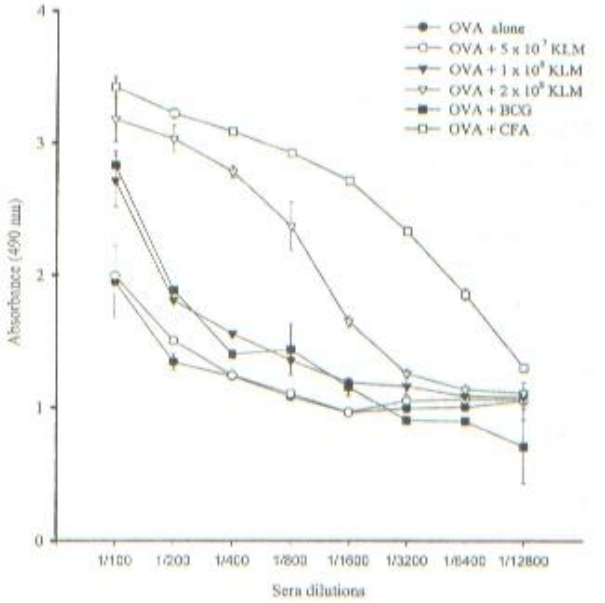

Fig. 2. Adjuvant effect of KLM on the anti-OVA IgG response. Groups of 10 mice were immunized at the base of the tail with OVA + different doses of KLM. Five mice from each group were boosted after one week with OVA alone. Twenty days later, sera were harvested and tested individually for the presence of anti-OVA IgG antibodies by ELISA. The values reported in the graph represent the mean of the absorbance values found for individual mice within one group; bars represent the standard deviation.

DISCUSSION

The use of BCG as an adjuvant is regarded as an acceptable practice in man, and at present this adjuvant is routinely used in vaccination and immunotherapy trials against leishmaniasis [12-16]. However, there is evidence that BCG inoculation may lead to the development of inflammatory arthritis, and it has been suggested that BCG immunotherapy may be a trigger mechanism for induction of autoimmune reactions [19]. In addition, the application of a live vaccine as adjuvant may cause complication [18], especially in immunocompromised individuals. Therefore, evaluation of the adjuvant effect of KLM may be of interest in view of establishing new strategies for vaccination or immunotherapy in leishmaniasis and other infections.

The results of the present study indicate that KLM does exert an adjuvant effect on the immune response to an unrelated soluble antigen, as the production of IFN-γ and serum antibodies. In several of these assays, the response obtained using KLM was comparable to that of achieved with more conventional adjuvants such as BCG or CFA. Interestingly, low levels of IL-4 were obtained in lymph node cell cultures from the mice injected with OVA + the lowest dose of parasites (5×10^7). The level of IL-4 in this group of mice was lower than that of seen in BCG injected mice, leading to the highest IFN-γ/IL-4 ratio observed in this experiment. These results are consistent with the idea that a low dose of KLM may promote a Th1 response. However, higher KLM dosages boosted the IL-4 response to levels comparable or higher than those seen with BCG or CFA.

Antibody measurements showed that the mice injected with OVA + 1×10^8 KLM developed titers similar to those of seen in the mice injected with OVA + BCG. Likewise, a marked increase in titer was demonstrated in the mice injected with OVA + 2×10^8 KLM, which was close to that of observed in the mice injected with OVA + CFA.

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The combination of KLM with BCG has been used in humans by several investigators, in assays aimed to assess both its protective effects as a vaccine [13-16] and its immunotherapeutical potential in *Leishmania* infections. The later strategy was originally devised by Convit *et al.* [12], reported that a percentage of leishmaniasis cases with abnormal immunological features responded well to this treatment. This approach was postulated to exercise its beneficial effect at least partly because BCG might promote better antigen presentation and stimulate a cellular immune response, via secretion of IL-2 and IFN-γ, leading to macrophage activation and intracellular parasite killing [12]. As shown in this report, *Leishmania* itself appears to be endowed with similar characteristics. In the murine model of *L. major* infection, it is now well established that protection depends on a cell-mediated immune response with expansion of a Th1 subset of lymphocytes [24, 25]. Similarly, in some human studies, it was shown that T-cell responses and production of IFN-γ in the early phase of infection were essential for the development of protective immune responses [26, 27]. Likewise, in vaccine trials in man, conversion of Montenegro test as well as IFN-γ production and absence of IL-4 secretion have been reported after injection of killed leishmanial antigen alone [13, 28, 29]. Based on the results of this investigation, the use of KLM promastigotes as an adjuvant should be considered in immunological studies, particularly in situations where the selective development of a Th1 response appears to be desirable.

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