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Analysis of the adjuvant effect of recombinant *Leishmania infantum* Hsp83 protein as a tool for vaccination

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Abstract

The properties of *Leishmania infantum* hsp83 (LiHsp83) to elicit an immune response against a fused reporter antigen, maltose binding protein (MBP), was studied. CF1 mice were immunized with different purified recombinant proteins: MBP, LiHsp83 and MBP fused to LiHsp83 (MBP-LiHsp83). Serum samples were obtained at days 0, 21, 28, 60, 90, 120 and 150 post-immunization. MBP-LiHsp83 fusion protein elicited a strong humoral response against MBP, higher than that one obtained in mice immunized with MBP alone or MBP mixed with LiHsp83, showing the secretion of both anti-MBP IgG2a and IgG1 isotypes (IgG2a/IgG1 ratio: 2:1). This response was specific for recombinant proteins and was maintained for at least 150 days, whereas the reactivity in mice immunized with MBP alone dissapeared at day 90. After in vitro stimulation with MBP, spleen cells from MBP-LiHsp83 immunized mice showed higher proliferation indices and produced higher secretion of IFN-γ than spleen cells from either control or MBP-immunized mice. In all groups of mice IL-4 was undetectable. Thus we consider that LiHsp83 may be a promising candidate to be used as carrier of fused antigens for adjuvant-free vaccination.

Keywords: Heat shock protein; Hsp83; Carrier; Adjuvant; Vaccine

1. Introduction

Heat shock proteins (hsps) are a family of proteins that are constitutively expressed at high level, and which increase further when a cell is subjected to stress conditions. Hsps are also highly conserved from bacteria to mammals and are involved in many essential cell functions as cause of their molecular chaperonin features: protein translocation, folding and assembly. Compelling evidence exits demonstrating that hsps of bacterial and parasitic pathogens are strong immunogens [1]. In some cases adjuvant effects of hsps have been assessed. *Mycobacterium tuberculosis* hsp60 and hsp70 proteins have been used to conjugate synthetic *Plasmodium falciparum*-derived-peptides, eliciting a long-lasting humoral response against the peptides [2]. Suzue and Young [3] have demonstrated that *M. tuberculosis* hsp70 enhance both humoral and cellular response against a recombinant HIV p-24 antigen when linked to the hsp carrier. Similar results were observed using a *Leishmania infantum* hsp70 fused to maltose binding protein (MBP) as reporter antigen [4]. In addition, it was shown that the ability to stimulate the humoral immune response lies in the amino terminal domain of the *L. infantum* hsp70, the most conserved region of this molecule [5].

Despite hsps gp96 and hsp90 associated with antigenic peptides triggers immune response against chaperoned-peptide [6,7], there is little evidence about the adjuvant effect of the hsp83-90 family. Preliminary results showed that *L. infantum* hsp83 (LiHsp83) also fires a strong humoral response to a fused protein, causing a mixed IgG1/IgG2a response [5]. The goal of the present study was to analyze the 'adjuvant' LiHsp83 as a tool for vaccination.

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2. Material and methods

2.1. Plasmids and recombinant proteins

Plasmids pMal-cRI, pM83 and pQE83 expressing the maltose binding protein (MBP), MBP fused to LiHsp83 (MBP-LiHsp83) and a 6-histidine tag fused to LiHsp83, respectively, have been described previously [4,8]. Recombinant proteins were expressed in M15 *Escherichia coli* strain. MBP and MBP-*Leishmania* proteins were purified by affinity chromatography on amylose resin (New England Biolabs) as described [10], and LiHsp83 was purified by non-denaturing conditions on nitrilotriacetic acid-Ni2+ column (Qiagen) as described [9]. The purity of recombinant proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels in the Mini-Protean system (Bio-Rad).

2.2. Immunizations

Two-month old female CF1 mice ($n = 10$ per group) were immunized intraperitoneally (i.p.) with each antigen on days 0 and 21. The immunization doses and boosters of each antigen were the following: MBP (42 kDa, 1 μg), LiHsp83 (86-kDa, 2 μg) and MBP-LiHsp83 (128 kDa, 3 μg). Mice were periodically bled from the retro-orbital plexus.

2.3. Determination of IgG titers and isotypes

Enzyme linked immunosorbent assay (ELISA) was performed as described [10] using 500 ng/well of recombinant proteins to coat the plates. Immune complexes were developed with OPD (Sigma) as the chromogen and H_2O_2 as the substrate of horseradish-peroxidase conjugated to anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) which was used as second antibody. Absorbance at 450 nm (A_{450}) was measured with an automatic ELISA reader (Dynatech MR4000). Each serum was tested in duplicate. At least two independent ELISAs were performed for each serum. Isotype-specific analysis were done by ELISA using the horseradish peroxidase conjugated anti-mouse IgG1 and IgG2a antibodies (Serotec), diluted 1:4000 and 1:2000, respectively.

2.4. Western-blot analysis

SDS-PAGE on 10% polyacrylamide gels was performed in the Mini-Protean system (Bio-Rad) using standard conditions. After electrophoresis, proteins were transferred to nitrocellulose membrane (Mini-Protean-Blot system, Bio-Rad), and the membrane was blocked with 5% non-fat dried milk powder in PBS-0.5% Tween 20 (blocking solution). The nitrocellulose filters were probed with the first antiserum diluted in blocking solution. A peroxidase immunoconjugate (Jackson ImmunoResearch Laboratories) was used as the secondary antibody (1:5000), and specific binding was detected with H_2O_2 as substrate and diaminobenzidine as the chromogen.

2.5. Analysis of MBP-dependent proliferation of spleen cells

In vitro proliferation assays were performed with RPMI culture medium supplemented with 10% fetal calf serum, 2-mercaptoethanol at a final concentration of 5×10^{-5} M, penicillin (100 U/ml) and streptomycin (100 μg/ml). Spleens from four immunized mice from each group were removed at day 120 after first immunization. Four phosphate buffer saline (PBS)-injected CF1 mice were added as control group. Viable spleen cells were plated by triplicate in 200 μl of medium at 3×10^5 cells per well in 96-well flat-bottom microcultures plates (Costar, Cambridge, MA). Cells were stimulated with optimal concentration of 10 μg of MBP. Positive controls were assayed with concanavalin A in all experiments. Culture medium alone was used for negative controls. One μCi/well of ^{3}H-thymidine (specific activity 5 Ci/mmol, Amersham Corp.) was added for 24 h. ^{3}H-thymidine incorporation was measured at 72 h in a LKB (Gaithersburg, MD) liquid scintillation counter. Results were expressed as stimulation index (SI): the mean of counts per minute (cpm) of MBP-stimulated cells divided by the mean of cpm from non-stimulated cells.

2.6. Cytokines

To measure IFN-γ and IL-4, spleen cell cultures (5×10^6 cells in 200 μl of medium) from infected mice were stimulated as mentioned above. Supernatants were harvested at 48 and 72 h and stored at −20°C until IFN-γ and IL-4 contents were measured by ELISA. Microtiter plates (Immuno Plate Maxisorp; Nunc) were coated overnight at 4°C with 3 μg/ml of the capturing rat anti-mouse-IFN-γ and IL-4 (Pharmigen). Supernatants were tested by triplicate and serial dilutions of recombinant IFN-γ and IL-4 (Pharmigen) proteins were used at 20–4000 pg/ml and incubated for 1 h at 37°C. After washes, 1 μg /ml of biotinylated rat anti-mouse-IFN-γ (Pharmigen) and biotinylated rat anti-mouse-IL-4 (Pharmigen) were added for 1 h at 37°C. After washes, extravidin-peroxidase conjugate (Sigma) diluted 1:1000 was added. Bound complexes were detected by an OPD (Sigma) reaction. ELISA results were determined for each serum in duplicate at 450 nm. At least two independent ELISAs were performed for each supernatants. IFN-γ content was calculated as picograms per milliliter using as reference the recombinant IFN-γ curves.

3. Results and discussion

3.1. Analysis of the humoral response of mice immunized with recombinant proteins

The presence of anti-MBP IgG and isotypes was evaluated over a period of 150 days in mice immunized with equimolar doses of MBP, MBP-LiHsp83, MBP mixed with LiHsp83 (MBP + LiHsp83) or LiHsp83. None of the immunizations included an adjuvant. Mice immunized with MBP-LiHsp83 elicited the highest anti-MBP IgG titer, averaging to 49 000 at day 28 (Fig. 1(A)). These titers descended to near 20 000 at day 60 and were maintained thereafter. Mice injected with MBP alone or MBP + LiHsp83 had also their highest anti-MBP IgG titers at day 28 averaging to 875 (Fig. 1(A)) and 1000 (data not shown), respectively, becoming negative at day 90. In conclusion, MBP linked to LiHsp83 elicited a humoral response greater than those observed in mice immunized with MBP alone or not linked to heat shock protein, similar as it was demonstrated to occur with hsp70s from *Mycobacterium* and *Leishmania* [3,4].

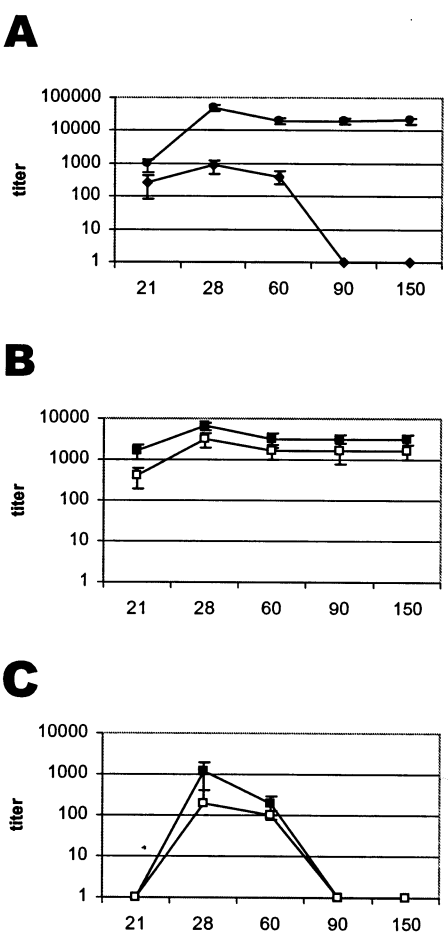

Fig. 1. Anti-MBP IgG and IgG isotypes profile of serum samples obtained from CF1 mice immunized with MBP and MBP fused to LiHsp83 proteins. Immunized mice were bled periodically. Data of serum samples obtained at days 21, 28, 60, 90 and 150 are represented. Titers were determined as the highest serum dilution giving absorbance value four times greater than the value obtained with preimmune sera. (A) Mean of anti-IgG titers of serum samples from mice immunized with MBP-LiHsp83 (circle), and MBP (square). (B–C) Anti-IgG1 (open square) and -IgG2a (filled square) titers from mice immunized with MBP-LiHsp83 (B) and MBP (C).

Fig. 1(B) showed that immunization of CF1 mice with fusion protein MBP-LiHsp83 elicited strong anti-MBP IgG2a and IgG1 antibody response, with a IgG2a/IgG1 ratio near 2 along the experiment. Immunization with MBP alone developed a predominant anti-MBP IgG2a isotype profile that decreased up to day 90 (Fig. 1(C)). Similar results were found for MBP + LiHsp83 (data not shown).

The anti-LiHsp83 reactivity of mice immunized with LiHsp83 and MBP-LiHsp83 recombinant proteins was determined at day 28. In all cases, serum samples assayed by IgG-ELISA showed titer values averaging 10^5. Mice immunized with MBP-LiHsp83 or LiHsp83 resulted in the presence of both anti-LiHsp83 IgG1 and IgG2a isotypes (data not shown). Sera from mice immunized with MBP-LiHsp83 did not show cross reactivity with proteins from murine foreskin fibroblast cell line as determined by immunoblot (Fig. 2, lane 2), indicating that autoantibodies were not induced by immunization with LiHsp83.

3.2. Cellular immune response induced by recombinant protein immunizations

MBP-dependent cellular immune response in recombinant protein-immunized mice was evaluated at day 90 post-immunization. Substantial specific lymphoproliferation was observed in spleen cell cultures obtained from MBP-LiHsp83-immunized mice after 72 h of culture (Fig. 3(A)). Proliferation of splenocytes from MBP-immunized mice was also high, but consistently lower than that obtained for splenocytes from MBP-LiHsp83 group.

Spleen cells from MBP-LiHsp83 immunized mice produced significant levels of IFN-γ when stimulated with MBP, compared to controls (Fig. 3(B)). IL-4 levels were low for all groups (< 20 pg/ml). These results are in agreement with those found for isotype analysis for CF1 mice immunized with MBP-LiHsp83, where anti-MBP IgG1 levels were lower than IgG2a.

Our results with LiHsp83 are promising and should be taken in consideration for developing of free-adjuvant vaccine systems. *L. infantum* hsp83 (LiHsp83) elicited a high and long lasting humoral and cellular immune response against the reporter antigen MBP, when co-administered as fusion protein. The association of IgG isotype and Th cell type is well defined in mice: an IgG1 antibody response is primarily driven by Th2, and IgG2a by Th1 T cells [11,12]. Mice immu-

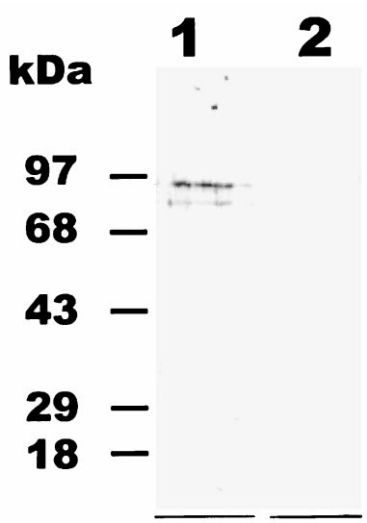

Fig. 2. Immunoblot of murine fibroblast foreskin cell proteins with rabbit anti-*Toxoplasma gondii* Hsp90 (lane 1) and MBP-LiHsp83 immunized mice (lane 2) sera. Sera were used at a dilution of 1:500 (lane 1) and 1:50 (lane 3). In lane 1, potential murine Hsp90 is detected. Molecular masses are given on left.

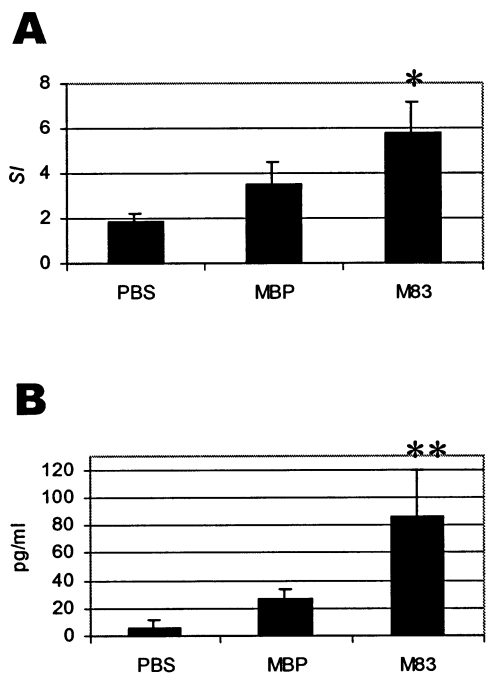

Fig. 3. Splenocytes from mice immunized with MBP, MBP-LiHsp83 and PBS (control) were stimulated in vitro with 10 μg of MBP. Four mice per group were analyzed. (A) lymphoproliferation analysis after 72 h of in vitro incubation represented as stimulation index (SI). SI of concavalin A stimulation ranged from 12.7 to 20.1. (B) IFN-γ production after 48 h of in vitro incubation. The significance of data was determined by Student's *t*-test. * and **, significantly higher ($P < 0.05$ and $P < 0.01$, respectively) than the MBP group.

nized with MBP-LiHsp83 elicited an anti-MBP IgG1/IgG2a response, inferring that both Th1 and Th2 responses were primed. In the present study, we demonstrated the production of specific IFN-γ (Th1 cytokine). During *Toxoplasma gondii* infection IFN-γ showed to be a major mediator against parasite infection [13]. At present, our interest is focused on the use of LiHsp83 as a carrier/immunostimulatory molecule for the Rop2 antigen [10], analyzing its potential capacity to stimulate an effector immune response against *T. gondii* in mice.

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