Characterization of the Immunostimulatory Properties of Leishmania infantum HSP70 by Fusion to the Escherichia coli Maltose-Binding Protein in Normal and nu/nu BALB/c Mice

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Leishmania infantum HSP70 has been described as an immunodominant antigen in both humans and dogs suffering from visceral leishmaniasis. In this study, we used L. infantum HSP70 fused to Escherichia coli maltose-binding protein (MBP), as the reporter protein, to analyze the influence of HSP70 on the immunogenicity of MBP in BALB/c mice. Plasmids were constructed to produce the three recombinant proteins used in this study, namely, MBP, L. infantum HSP70, and MBP-HSP70, which consists of MBP fused to the L. infantum HSP70 amino terminus. Immunization of BALB/c mice with the MBP-HSP70 fusion protein elicited humoral and cellular responses against MBP that were higher by an order of magnitude than those elicited by immunization with MBP alone or with a mixture of MBP and HSP70. Covalent linkage of MBP to HSP70 was essential for eliciting a strong anti-MBP immune response. Cytokine secretion and immunoglobulin G isotype analyses indicated that immunization with the MBP-HSP70 fusion protein preferentially induces a Th1 immune response. Immunization of athymic nu/nu mice with the MBP-HSP70 fusion protein unexpectedly gave rise to an anti-MBP humoral response showing features of a T-cell-dependent response. Thus, we present evidence that L. infantum HSP70 demonstrates an adjuvant effect in the immune response against a covalently linked reporter protein.

The heat shock proteins (HSP) are produced by prokaryotic and eukaryotic cells in response to a variety of physiological insults. Those HSP belonging to the HSP90, HSP70, and HSP60 families are among the most highly conserved and abundant proteins found in living prokaryotic and eukaryotic organisms. Although they are classified as stress proteins, the HSP also have essential functions in the cell under normal growth conditions. These proteins are involved in the folding of newly synthesized proteins by preventing incorrect interactions within and between nonnative proteins (reviewed in reference 18). The HSP are also involved in several molecular processes of the immune system, such as immunoglobulin chain assembly (17), antigen processing and presentation (29), and the assembly of functional major histocompatibility complexes I and II (12). Although the abundance of the HSP may explain why they behave as dominant antigens in the immune response to a variety of pathogens (25, 26, 35), it is surprising that the immune system focuses its attention on a family of proteins with such a high degree of evolutionary conservation. It has been suggested that immune recognition of HSP of pathogens serves as a first line of defense as well as a source of autoimmune cascades that may develop through inappropriate cross-reactivity with self-HSP (8, 40). Of the HSP families, HSP70, in spite of being the most conserved protein, present in all organisms studied to date (16), is a major immunogen in infections caused by a number of pathogens, such as Schistosoma mansoni (19), Onchocerca volvulus (34), Plasmodium falciparum (2, 4, 5, 21), Histoplasma capsulatum (1), Trypanosoma cruzi (14, 23, 32), Trypanosoma congolense (6), and Leishmania (11, 24, 30, 36) and Mycobacterium (9, 15, 27, 33) spp. Epitope analysis of several of these HSP70 indicates the existence of multiple B- and T-cell epitopes throughout their primary structure (4, 13, 28, 31, 32, 41).

The immunogenic properties of the HSP have been demonstrated in particular for Mycobacterium tuberculosis HSP70, which has been used successfully as an adjuvant-free carrier molecular (3). Recently, Suzue and Young (38) have further investigated the immunogenic potential of M. tuberculosis HSP70 by immunizing mice with a recombinant human immunodeficiency virus type 1 p24-HSP70 fusion protein. They demonstrated that in the absence of adjuvants, covalent linking of HSP70 to p24 was essential to elicit humoral and cellular immune responses to p24. Here, we have addressed the question of whether the immunostimulatory property of M. tuberculosis HSP70 is possessed by other HSP70. Based on the powerful immunogenic characteristics of Leishmania infantum HSP70 (30, 31), we analyzed whether L. infantum HSP70, when fused to an accompanying protein, can induce humoral and cell-mediated responses to the fused protein.

Plasmid constructions, purification of proteins, mice, and immunizations. DNA coding for L. infantum HSP70 was amplified by PCR as described elsewhere (30). The amplification product was digested with EcoRI and HindIII restriction enzymes, purified on spin bind columns (FMC Bio Products, Rockland, Maine), and cloned in frame to the carboxyl terminus of the maltose-binding protein (MBP) encoded by the vector pMAL-cRI (New England Biolabs, Inc., Beverly, Mass.). The resulting clone was named pMAL-rLiHsp70. To obtain the recombinant protein MBP alone, the pMAL-cRI plasmid was digested with EcoRI, filled in with Klenow fragment, and religated with T4 DNA ligase. Consequently, the
modified vector, pMAL-cRI*, includes an in-frame stop codon four triplets downstream of the EcoRI restriction site. The pMAL-cRI* vector thus expresses the MBP moiety alone rather than the MBP-lacZa expressed by the original pMAL-cRI plasmid. The pMAL-cRI* was digested with EcoRI and HindIII, the insert was cloned in the corresponding restriction sites of the vector pBluescript (Stratagene, La Jolla, Calif.), and the resulting clone was denominated pBls70L. Clone pQEM70 was created by subcloning the BamHI-HindIII insert of plasmid pBls70L into the appropriate restriction sites of vector pQE9 (Qiagen, Hilden, Germany).

Induction and purification of the recombinant proteins expressed by clones pMAL-LiHsp70 and pMAL-cRI* were performed as described in the manufacturer's instructions (New England Biolabs). The recombinant protein _L. infantum_ HSP70, expressed by clone pQE/hsp70, was purified under denaturing conditions with a nitrilotriacetic acid-Ni²⁺ column (Qiagen). Figure 1A shows a schematic representation of the recombinant proteins used. The purity of the recombinant proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining (Fig. 1B).

Female 12-week-old BALB/c mice and female 7-week-old, athymic BALB/c nu/nu mice were purchased from IFFA-CREDO (Lyon, France). The immunization schedule was the same as that described by Suzue and Young (38), i.e., mice were immunized intraperitoneally (i.p.) on days 0 and 21 with 50 pmol of the purified protein in 200 μl of phosphate-buffered saline, which corresponds to 2.1, 3.5, and 5.6 μg of MBP, HSP70, and the MBP-HSP70 fusion protein, respectively. The mice were periodically bled from the retro-orbital plexus.

_L. infantum_ HSP70 potentiates a specific humoral response against the covalently linked antigen. By using the data of Suzue and Young (38), we studied whether _L. infantum_ HSP70 can elicit an immunostimulatory effect when used as a carrier molecule in adjuvant-free immunizations of mice. _Escherichia coli_ MBP was chosen as the reporter antigen. Plasmid constructions encoding MBP, _L. infantum_ HSP70, or the MBP-HSP70 fusion protein were obtained (Fig. 1). Groups of four BALB/c mice were immunized with MBP, HSP70, an MBP-HSP70 mixture, or the MBP-HSP70 fusion protein. One week after the last immunization, the anti-MBP immunoglobulin G (IgG) level in serum was determined with the FAST-ELISA (Becton Dickinson, Lincoln Park, N.J.) by using the coating antigen at a concentration of 2 μg/ml. Horseradish peroxidase-conjugated anti-mouse IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) was used as the second antibody at a 1:2,000 dilution. The titer is expressed as the highest serum dilution giving an absorbance value four times higher than the preimmune serum reading (optical density, 0.050).

Differences in antibody titers between groups of mice were compared with the two-tailed unpaired Student t test. Excel software (Microsoft Corporation) was used for statistical analysis.

The anti-MBP IgG serum titers among mice immunized with MBP alone varied significantly, ranging from 8,000 to 256,000 (mean value = 1.3 × 10⁵; standard deviation [SD] = 10⁴) (Fig. 2A). Similar serum titers and variations were found for serum samples from mice immunized with the MBP-HSP70 mixture (mean value = 2.2 × 10⁵; SD = 1.8 × 10⁴); this was an indication that the presence of HSP70 in the mixture did not influence the antibody response elicited by the MBP. The anti-MBP titers (2 × 10⁵; SD = 10²) detected in sera from mice immunized with the MBP-HSP70 fusion protein were approximately 1 order of magnitude greater than those observed in sera from mice immunized with MBP or the MBP-HSP70 mixture. It was observed, moreover, that while the titers of the anti-MBP humoral response in animals immunized with the protein alone or with the MBP-HSP70 mixture were highly variable, the anti-MBP titers in MBP-HSP70 fusion protein-immunized animals were uniform. It thus appears that _L. infantum_ HSP70 can function, in the absence of an adjuvant, as an adjuvant-carrier molecule and can induce high antibody levels to the HSP70-accompanying protein; this effect occurs only when MBP is covalently linked to HSP70.

To determine whether HSP70 may also condition the nature of the anti-MBP humoral response, the anti-MBP IgG1 and IgG2a isotypes resulting from administration of MBP, the MBP-HSP70 mixture, and the MBP-HSP70 fusion protein were analyzed (Fig. 2B). Th1 cytokines preferentially elicit an IgG2a antibody response, whereas Th2 cytokines stimulate IgG1 (7). The anti-MBP IgG1 and IgG2a titers induced by MBP immunization were not significantly different (P = 0.308), with values of 5 × 10⁴ (SD = 3.6 × 10⁴) and 2.7 × 10⁴ (SD = 2.5 × 10⁴), respectively; the anti-MBP IgG1 and IgG2a titers in mice receiving the MBP-HSP70 mixture were also similar (P = 0.665), with values of 6.0 × 10⁴ (SD = 4.1 × 10⁴) and 4.5 × 10⁴ (SD = 5.4 × 10⁴), respectively. However, in mice immunized with the MBP-HSP70 fusion protein, the IgG2a response was consistently higher than that of IgG1 (P < 0.0001), with anti-MBP titers of 2 × 10⁵ (SD = 1.0 × 10⁴) and 8.1 × 10⁴ (SD = 4.0 × 10⁴), respectively. Thus, it seems that following the injection of the MBP-HSP70 fusion protein, there is a switch of anti-MBP antibodies towards an IgG2a isotype.

The time course of anti-MBP IgG titer evolution was monitored over a 17-week period in the three groups of immunized
were reimmunized 20 weeks after the initial immunization with 50 pmol of the same MBP-HSP70 fusion protein preparation. One week after administration of the recombinant protein, anti-MBP IgG titers increased by 1 order of magnitude, reaching values of $4.5 \times 10^6$ (SD = $2 \times 10^5$). This rapid and potent response reflects the immunological memory created by immunization with the MBP-HSP70 antigen.

**Immunization with the MBP-HSP70 fusion protein induces a powerful cellular response against MBP.** To examine the ability of the MBP-HSP70 fusion protein to induce anti-MBP cellular responses, groups of four BALB/c mice were immunized subcutaneously at the tail base with 5 µg of the MBP-HSP70 fusion protein, MBP, or bovine serum albumin (BSA; Sigma, St. Louis, Mo.). One week after immunization, draining inguinal and periaortic lymph nodes were removed, and single-cell suspensions of lymph node cells (LNC) were prepared and suspended in complete medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 10 µM 2-mercaptoethanol). LNC (10^6/well) were dispensed into 96-well microtiter plates. Cultures were challenged in triplicate with 2 µg of MBP or concanavalin A (ConA; Sigma) per ml and incubated for 3 days at 37°C in 5% CO₂; after this, 1 µCi of [³H]thymidine (5 Ci/mmol; Amersham Corp., Aylesbury, United Kingdom) was added to each well. After 16 h, the cells were harvested and [³H]thymidine incorporation into DNA was measured by liquid scintillation counting. The specific incorporation was determined by subtracting the mean incorporation of triplicate control wells incubated with medium alone from the mean incorporation of the triplicate wells incubated with antigen. Table 1 shows the MBP-specific immune response of each group following in vitro T-cell stimulation with MBP. LNC from animals immunized with the MBP-HSP70 fusion protein proliferated in response to the antigen, whereas those from animals immunized with MBP alone did not. The proliferation levels of LNC from MBP-immunized animals were similar to those of LNC from control animals to which BSA had been administered (Table 1). The ability of the fusion protein to elicit cellular responses was also examined by determination of gamma interferon (IFN-γ), interleukin 2 (IL-2), and IL-4 cytokines from LNC supernatants (Table 1).

Cytokine determinations, LNC (5 x 10⁶/well) from the same mice were seeded in 24-well plates and the cultures were challenged with 2 µg of MBP or ConA per ml for 72 h at 37°C in 5% CO₂. IFN-γ, IL-4, and IL-2 production was measured in supernatants with an enzyme-linked immunosorbent assay kit (Genzyme Corp., Cambridge, Mass.). Results of the cytokine determinations are shown in Table 1.

**TABLE 1. Proliferation and cytokine production of LNC**

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Stimulus</th>
<th>[³H]thymidine incorporation (cpm [mean ± SD])</th>
<th>Concentration (pg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IFN-γ, IL-2, IL-4</td>
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<tr>
<td>BSA</td>
<td>Medium</td>
<td>2,355 ± 350</td>
<td>&lt;20 &lt;15 &lt;20</td>
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<td></td>
<td>ConA</td>
<td>50,313 ± 2,246</td>
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<td></td>
<td>MBP</td>
<td>4,459 ± 194</td>
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</tr>
<tr>
<td>MBP</td>
<td>Medium</td>
<td>3,378 ± 844</td>
<td>&lt;20 &lt;15 &lt;20</td>
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<tr>
<td></td>
<td>ConA</td>
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<td></td>
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<td>4,023 ± 746</td>
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<tr>
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<td>11,220 ± 1,462</td>
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<td></td>
<td>ConA</td>
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<tr>
<td></td>
<td>MBP</td>
<td>50,589 ± 4,877</td>
<td>125 75 20</td>
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*The final concentrations of ConA and MBP were 2 µg/ml.

*Mean ± SD of triplicate determinations.*

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FIG. 2. Humoral immune response of BALB/c mice to different recombinant proteins. (A) Groups of four mice were immunized i.p. with MBP, an MBP-HSP70 mixture, or the MBP-HSP70 fusion protein as indicated in the text. The anti-MBP and anti-HSP70 IgG antibody titers were determined with the FAST-ELISA. The titer is expressed as the highest serum dilution factor giving an absorbance value four times greater than the value obtained with preimmune sera (optical density, 0.05). (B) Serum samples from mice immunized with MBP, the MBP-HSP70 mixture, or the MBP-HSP70 fusion protein were analyzed for anti-MBP IgG1 and IgG2a antibodies. (C) The time course of the anti-MBP antibody response is shown. Sera were obtained at the times indicated (in weeks) after the second immunization of the mice with MBP, the MBP-HSP70 mixture, or the MBP-HSP70 fusion protein. MBP + HSP70, mixture; MBP-HSP70, fusion protein.
membranes (Amersham). The filters were blocked with 5% nonfat dried milk powder in phosphate-buffered saline–0.5% Tween 20 and probed sequentially with primary and secondary antisera in blocking solution. A peroxidase immunoconjugate (Nordic Immunological Laboratories) was used as a secondary antibody, and specific binding was developed with 0.5 mg of 4-chloronaphthol (Sigma) per ml and 0.025% hydrogen peroxide.

Proteins were probed in Western blots with either rat monoclonal antibody 7.10 (Fig. 3B), which recognizes HSP70 independently of its origin (39), serum from a mouse immunized with the MBP-HSP70 mixture (Fig. 3C), or serum from a mouse immunized with the MBP-HSP70 fusion protein (Fig. 3D). Anti-HSP70 antibodies induced by immunization with L. infantum HSP70 (alone or as a fusion protein) show no cross-reactivity with HSP70 of mammalian origin. This selectivity of response is also evident in the course of natural L. infantum infection, since it has been observed that HSP70, one of the immunodominant antigens in Leishmania infection, elicits highly specific antibodies only to the parasite protein (30). The cross-reactivity with T. cruzi HSP70, present in some sera (Fig. 3D), can be explained by the high degree of sequence conservation between Leishmania and T. cruzi proteins (30). Cross-reactivity with T. cruzi HSP70 was observed in 75% of the serum specimens from mice immunized with the MBP-HSP70 fusion protein (data not shown).

Immunization with the MBP-HSP70 fusion protein induces a humoral response in athymic BALB/c nu/nu mice. To study T-cell involvement in the MBP-HSP70 fusion protein-induced humoral response, athymic BALB/c nu/nu mice were immunized twice with MBP, HSP70, or the MBP-HSP70 fusion protein. A positive anti-MBP antibody response was detected in nu/nu mice only after immunization with the MBP-HSP70 fusion protein (Fig. 4). The humoral response elicited in these mice was a typical, T-cell-dependent response. Following the first immunization, an IgM isotype was detected, whereas IgG predominated after challenge (Fig. 4B). The anti-MBP antibodies elicited in the fusion protein-immunized mice were essentially IgG2a (antibody titers, >1,200). Immunization of nu/nu mice with MBP or HSP70 elicited no humoral response (Fig. 4).

The adjuvant effect of HSP70 was first demonstrated after immunization with Plasmodium peptides or meningococcal oligosaccharides cross-linked to M. tuberculosis HSP70 (3). Our results show that this carrier-adjuvant effect is also developed by L. infantum HSP70. Another example of this adjuvant effect is the anti-p53 antibody response in cancer patients, which is elicited only if mutated p53 is complexed with HSP70 in tumor cells (10). Thus, it seems that the adjuvant effect may be a common feature of this class of proteins. This phenomenon can be related, at the molecular level, to the tumor-specific immunity elicited by vaccination with HSP isolated from cognate cancer cells (37).

Two interesting features can be deduced from the analysis of the anti-HSP70 humoral response elicited in the mice immunized with L. infantum HSP70. First, the anti-HSP70 antibodies were directed specifically against L. infantum HSP70 epitopes. Such restriction in epitope selection would evidently be desirable if L. infantum HSP70 was to be used in adjuvant-free vaccination. Second, the anti-HSP70 antibody titer was clearly lower than the anti-MBP antibody titer in fusion protein-immunized mouse sera. It can be postulated that the humoral response to L. infantum HSP70 is partially restricted, perhaps through mechanisms involved in regulation of responses to self components (8). These features, together with the facts that the humoral response elicited by the MBP-
HSP70 fusion protein was long-lived, that the BALB/c mice exhibited a rapid and strong response after the boost, and that the response was observed in T-cell-immunodeficient mice, make the use of *L. infantum* HSP70 fused to specific antigens an attractive alternative to the use of adjuvants in the development of protective tools against infectious diseases.

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