

Conformational Stability and Antibody Response to the 18kDa Heat-Shock Protein Formulated into Different Vehicles

M. H. B. COSTA,^{*1} O. A. SANT'ANNA,²
P. S. DE ARAUJO,³ R. A. SATO,¹ W. QUINTILIO,¹
L. V. N. SILVA,¹ C. R. T. MATOS,¹ AND I. RAW¹

¹Laboratório de Microesferas e lipossomos- Centro de Biotecnologia;
and ²Laboratório de Imunogenética- Instituto Butantan, Avenida Vital Brasil,
1500, 05503-900, Butantan; and ³Departamento de Bioquímica,
Instituto de Química, USP, São Paulo S.P., Brasil

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ABSTRACT

Protein stability is one of the most important obstacles for successful formulation in the development of new-generation vaccines. Here, the 18kDa heat-shock protein (18kDa-hsp) was chemically modified through conjugation with bovine serum albumin or by esterification with *N*-hydroxysuccinimide ester of palmitic acid. The biologically active conformation of the protein was preserved after chemical modification. The immune responses to the recombinant 18kDa-hsp from *Mycobacterium leprae* were studied in different presentations: free, copolymerized with bovine serum albumin in aggregates (18kDa-hsp-BSA), and either surface linked to liposomes or entrapped into liposomes. Measuring the antibody production of immunized genetically selected mice has compared the adjuvant effects of liposomes and proteic copolymer. Among the two liposome preparations, the strongest response was obtained with the surface-exposed antigen-liposomes. The copolymer 18kDa-hsp-BSA conferred a high titer of antibody in injected mice, and persisted 70 d after immunization. This approach should prove very useful for designing more effective vaccines by using 18kDa-hsp as carrier protein.

*Author to whom all correspondence and reprint requests should be addressed. E-mail: mhbcosta@pasteur.fr.

Index Entries: Protein stability; protein carrier; heat-shock protein; vaccine adjuvants; supramolecular aggregates; liposomes.

INTRODUCTION

The investment in biotechnology over recent decades has resulted in the second generation of molecularly defined vaccines by using synthetic peptides, recombinant antigens, or extremely purified subunits (1–5). These subunit vaccines are potentially safer than those comprised of bacteria or dead attenuated viruses (6,7), although some of them are poorly immunogenic compared with those consisting of total virus or bacteria.

The poor activity of purified antigens is related to the adjuvant action of the components from virus or bacterial membrane. New approaches to improve the activity of molecularly defined vaccines such as recombinant live vaccine (8), new formulations for adjuvants (9), and new carrier molecules for peptides, proteins, or polysaccharides (10) have been described.

The induction of an efficient humoral response against a B-epitope requires the presence of an associated T-epitope (11). Traditionally, the source of T-epitopes are proteins such as tetanus toxoid, exotoxin from *Pseudomonas aeruginosa*, diphtheria toxoid, cross-reacting materials, pneumolysin, and some proteins from BCG or leprae, which have all been covalently bound to weak immunogens (12–15). The 18-kDa protein from *Mycobacterium leprae* (18kDa-hsp) is structurally close to heat-shock proteins (16) known to be involved in immunity. It can, potentially, be used as carrier protein (17). Furthermore, the 18kDa-hsp has sequences that can bind to B and T cells, and may therefore assure correct presentation of the bounded antigen to induce B- or T-cell immunity (18,19). As the highly purified recombinant 18kDa-hsp can be produced (20) at low costs (21), it was evaluated to determine if this protein resists chemical modifications for use as a carrier molecule. The advantage of vehiculation into delivery systems as liposomes is another tool to be explored.

Liposomes improve immune response to the antigen-injected animals (22) and are relatively nontoxic and nonimmunogenic (23). For instance, depending on their solubility and size, antigens can be accommodated within the aqueous spaces between bilayers, inserted in the lipid phase, or presented on the vesicle surface. Multiple-antigen accommodation into one or more of these locations is also possible. So, the system has the advantage of being able to incorporate such agents as immunomodulators that further improve immune responses (24).

Here we study the activity of the 18kDa-hsp after chemical modification and compare the antibody responses of the infected line of Selection GP (25,26) mice to exposed or encapsulated 18kDa-hsp into liposomes, or copolymerized with bovine serum albumin (BSA).

MATERIALS AND METHODS

Mice

H line from Selection GP (25,26) 2–4 mo-old mice of both sexes were used. These mice were maintained at the Laboratório de Imunogenética do Instituto Butantan.

Chemicals

Phosphatidylcholine (PC), dicetylphosphate (DCP) were kindly provided by Iolanda Midea Cuccovia, from Instituto de Química, Universidade de São Paulo. 3,3', 5,5'-tetramethylbenzidine (TMB), dimethylsulfoxide (DMSO), glutaraldehyde, bicinehoninic acid, cholesterol (Cho), deoxycholate, BSA, fraction V, antimouse-peroxidase conjugate were purchased from Sigma (St. Louis, MO). Sepharose 2B, Sepharose 4B, were purchased from Pharmacia (Uppsala, Sweden). Sunflower oil, salts, and solvents of analytical grade, were used without prior purification. The recombinant 18kDa-hsp was purified according to our previous description (20). The monoclonal antibody L5, that recognizes the 18kDa-hsp, was a gift from C. A. Moreira Filho, from I. C. B. of Universidade de São Paulo.

Preparation of Liposomes Containing the 18kDa-hsp

Multilamellar vesicles of phosphatidylcholine:dicetylphosphate:cholesterol (PC:DCP:Cho, respectively) in a molar ratio of 22.5:3.3:3.2 were obtained by dispersion of a lipid film in 10 mM citrate buffer, pH 6.0. Encapsulation of the 18kDa-hsp was carried out in the course of liposome formation (27,28). Surface-adsorbed protein was removed by trypsin followed by purification on a Sepharose 4B column, previously equilibrated with 10 mM citrate buffer, pH 6.0. Turbidity (400 nm) (29), protein (30), phosphorus (31), and ELISA (32) determinations monitored liposome elutions.

Preparation of Liposome Containing Externally Associated Antigen

The *N*-hydroxysuccinimide ester of palmitic acid (20 µg) was dissolved in 0.5 mL 97 mM deoxycholate in 10 mM citrate, pH 6.0 buffer, followed by incubation with 5 mg of 18kDa-hsp for 18 h (27). The solution of derivatized protein (Nacyl-18kDa-hsp) was shaken carefully with pre-formed liposomes (PC:DCP:Cho, 18:2:5) until the dispersion became clear. The *N*-acyl-18kDa-hsp-associated liposomes were purified by chromatography on a Sepharose 4B column, previously equilibrated with 10 mM citrate buffer, pH 6.0. Liposome elution was followed by turbidity (29), protein (30), phosphorus (31), and ELISA (32) assays.

Preparation of Protein Supramolecular Aggregates

The 18kDa-hsp (5 mg) and BSA (200 mg) were added to 1.25% glutaraldehyde (33) in phosphate buffer, pH 7.5 with stirring and immediately dispersed and injected into 100 mL sunflower oil and petroleum ether. The emulsion was magnetically stirred at constant rate. After 1 h at room temperature, it was washed three times with petroleum ether and purified on a Sepharose 2B column previously equilibrated with phosphate buffer. The supramolecular aggregates (or beads) elution was monitored by ELISA (32), turbidity (29), and protein determinations (30).

The 18kDa-hsp in vitro Release Study

The copolymers of 18kDa-hsp-BSA were applied on a Sepharose-4B column immediately after being produced. Aggregate fractions were stored at 4°C and after 13 and 21 d they were applied on a gel filtration column. Turbidity (29), protein (30), and ELISA (32) determinations followed elution profiles.

Immunizations

The mice were immunized with:

1. 50 µg of either free 18kDa-hsp or free BSA as controls.
2. 50 µg of the 18kDa-hsp entrapped into liposomes.
3. 15 µg of the N-acyl-18kDa-hsp-associated liposome.
4. 50 µg of the 18kDa-hsp-BSA aggregates, molar ratio of 0.09.

The mice were bled from the retro-orbital venous plexus for 10 weeks and the individual antibody titers (whole immunoglobulins) measured by ELISA (32).

ELISA

Liposomes and Proteic Aggregates

The liposomes or the proteic aggregates 18kDa-hsp-BSA were added to the ELISA plates and after 2 h at 37°C; they were blocked with 0.1% BSA for liposomes or with 0.1% horse serum albumin for the proteic aggregates. After 30 min L5, the monoclonal antibody against the 18kDa-hsp was added. The antimouse-peroxidase conjugate was added 30 min later and 3,3',5,5'-tetramethylbenzidine (TMB) was also added. After 15 min at room temperature, the absorbance was read at 450 nm.

Antibodies Antiliposomes Containing the 18kDa-hsp Titration

The 18kDa-hsp was added to the ELISA plates and was blocked with 0.1% horse serum albumin 2 h later. After 30 min the mice sera against

liposome-entrapped 18kDa-hsp or the sera antiliposomal-*N*-acyl-18kDa-hsp obtained from the immunized mice was added. The antimouse-peroxidase conjugate was added 30 min later and then TMB was added. After 15 min at room temperature the absorbance was read at 450 nm.

Antibodies Anti-18kDa-hsp-BSA Aggregates Titration

Free BSA, or free 18kDa-hsp were added to the plates than incubated at 37°C for 2 h and blocked with 0.1% horse serum albumin for 30 min. After blocking, immunized mouse serum was added and incubated for 30 min, followed by addition of conjugated antimouse-peroxidase and TMB. After 15 min at room temperature, the absorbance was read at 450 nm. The Titertek Multiskan MCC/340 was used for all lectures. All reactions were stopped with 1.17 M H₂SO₄. These results, obtained as absorbance, were converted in antibody titers.

Antibody Titers

Each mouse polyclonal serum was serially diluted (1:2 until 1:256). Antibody titers were expressed as $\log_2 \delta$, where δ is the reciprocal serum dilution factor giving an absorbance value of 20% of the saturation value.

RESULTS

Both multilamellar liposomes containing the entrapped or the externally associated 18kDa-hsp were polydispersed in size, eluting mainly at the void volume (*V*_o) of the Sepharose-4B column (not shown). The fractions containing the entrapped 18kDa-hsp or the externally associated *N*-acyl-18kDa-hsp were pooled and used in the immunization assays. As demonstrated by ELISA, the monoclonal antibody, L5 that is specific for the T-epitope in the 18kDa-hsp, recognized this site in both formulations. It means that the biologically active protein conformation was preserved.

It was observed through in vitro release study that the binding activity of 18kDa-hsp toward the monoclonal antibody L5 was not lost by the proteic aggregate production (Fig. 1A) or their release (Fig. 1B, C). This was monitored by ELISA. Any 18kDa-hsp activity could be determined at elution volumes of small particles, indicating that it was fully conjugated into large aggregates that eluted at the *V*_o (Fig. 1A). The releases of small particles containing the 18kDa-hsp and the turbidity decay at *V*_o were observed 13 (Fig. 1B) and 21 d (Fig. 1C) after their copolymerization with BSA.

The strongest antibody responses were related to the externally associated *N*-acyl-18kDa-hsp. The main kinetic pattern difference was that, whereas the exposed antigens induced a high response that decays with time, the entrapped ones began lower than the control group and

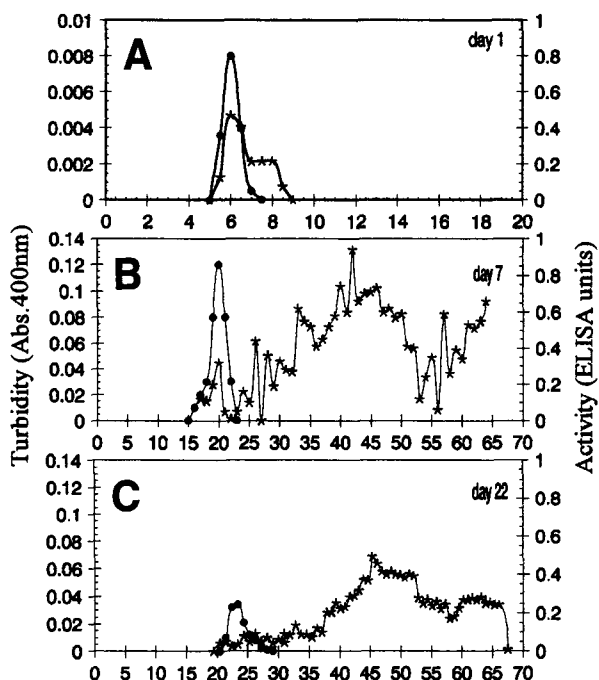


Fig. 1. Elution of the 18kDa-hsp-BSA aggregates on Sepharose columns, previously equilibrated with phosphate buffer, pH 7.5. Freshly prepared aggregates were applied on the column (total volume column of 20 mL) (A). The release of the antigen was followed by successive gel filtration (total volume column of 70 mL) at 13 (B) and 21 d (C). Elutions were followed by protein (data not shown), turbidity (●), and ELISA (*).

increased with time (Fig. 2). Both liposomal preparations were efficient in inducing a long-lasting response, with high antibody titers lasting 70 d after immunization. As demonstrated by ELISA, these polyclonal antibodies tested were also capable of recognizing the 18kDa-hsp.

Mice injected with free 18kDa-hsp presented a higher antibody level, in comparison with those injected with free BSA (Fig. 3). The antibodies produced against 18kDa-hsp-BSA aggregate was higher than the free 18kDa-hsp or the free BSA (Fig. 3). This means that the conjugation process was advantageous for both proteins. It is interesting to note that the specificity of the immune response was maintained, as the polyclonal anti-18kDa-hsp-BSA aggregate produced recognized both the free 18kDa-hsp or BSA as monitored by ELISA (Fig. 3).

DISCUSSION

The main goal of this work was to develop mild conditions to modify the potential carrier 18kDa-hsp and compare different processes to vehiculate this protein without immunological damage.

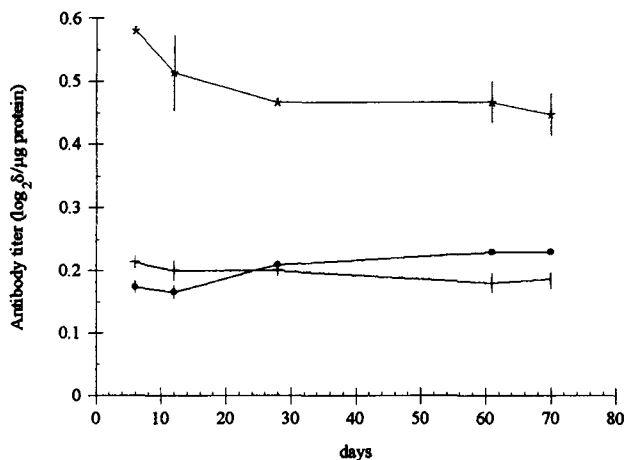


Fig. 2. Titers of polyclonal anti-18kDa-hsp entrapped into liposomes (●), or anti-*N*-acyl-18kDa-hsp associated liposomes (-*-) and anti-18 kDa-hsp (+). Mice were injected as described in subheading Materials and Methods. The antibodies production titers were expressed as $\log_2 \delta/\mu\text{g}$ proteins. (See definition for δ in the Materials and Methods section).

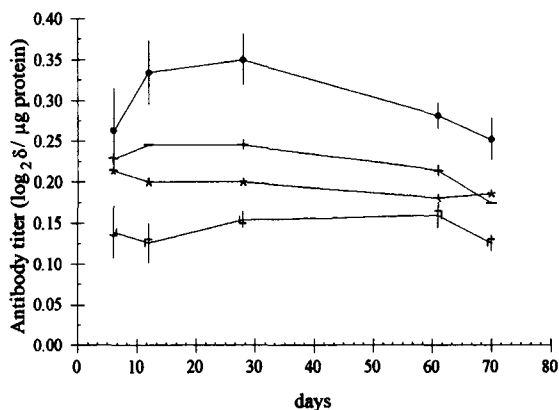


Fig. 3. Titers of polyclonal antibodies produced by the mice immunized with the aggregate 18kDa-hsp-BSA against time. The production of anti-18kDa-hsp or anti-BSA in this polyclonal anti-aggregate was tested by coating the ELISA plate with 18kDa-hsp (-+-) or with BSA (●). Controls: mice were immunized with free 18kDa-hsp or free BSA. The antibody produced against the free 18kDa-hsp (-*-), or against free BSA (-□-) were measured by ELISA. The antibodies production titer was expressed as $\log_2 \delta/\mu\text{g}$ protein (See definition for δ in the Materials and Methods section).

Liposome strongly potentiates the immune response and can also orient this response if properly manipulated (34) by using antigen encapsulation or surface linkage (34). Surface exposition can be achieved by a number of methods; such as covalent attachment of the antigen to one phospholipid to be used, by nonspecific adsorption of hydrophobic antigen with the phospholipid bilayer (35,36), or as demonstrated here, by

chemical modification of hydrophilic antigens. The 18kDa-hsp protein does not have any S-S (37) bond to sustain a rigid structural tertiary organization. Therefore, its five lysine residues could be derivatized (27,38), introducing, at least five hydrophobic tails through NH₃-Lys side chain, forming *N*-acyl-18kDa-hsp. The derivatization of the 18kDa-hsp and its external association to liposomal membrane did not alter the T-epitope conformation measured by ELISA determinations of the liposome preparations. The *N*-acyl-18kDa-hsp association with liposomes was 98%, corroborating literature data for other proteins (27). The results presented here demonstrated that the 18kDa-hsp masked by encapsulation or exposed to the milieu influenced the immune response with different kinetics (Fig. 2), in agreement with published data (39,40). Encapsulated 18kDa-hsp into liposome stimulated a long-lasting response (Fig. 2). In contrast, externally associated *N*-acyl-18kDa-hsp, initially induced the highest antibody level with slow decay (Fig. 2), remaining high for 70 d. Its immunogenicity was higher than control or the entrapped one. Furthermore, all of the mice sera recognized the unmodified 18kDa-hsp protein as determined by ELISA (Fig. 2).

A wide range of compounds with different chemical and physical properties can be entrapped in the BSA matrix (33) derived from the mild conditions used for formation of beads. Briefly the reaction utilizes glutaraldehyde as cross linker (41) forming a complex matrix (33). Probably the matrixes are formed through intra- and intermolecular linkages between glutaraldehyde and ϵ -NH₂ group of lysine (33), result of an intricate grid of Schiff bases. We exploit here the use of the mixed proteic matrix of BSA-18kDa-hsp. The most straightforward data is that the 18kDa-hsp epitope was preserved during the microsphere production attesting for the mild conditions of the conjugation process (10,33; Fig. 1).

The 18kDa-hsp small protein presented an immunogenic activity higher than the BSA that has 67 kDa. In contrast to 200 μ g BSA, a few micrograms of the 18kDa-hsp (5 μ g) in the formulation promoted high antibody levels. As the 18kDa-hsp/BSA molar ratio was low (0.09), we conclude that the immunogenicity of the proper 18kDa-hsp was superior to the BSA and may also result from their intrinsic properties. Here, the results from in vivo study indicated that the immunogenic response to the proteic aggregates, expressed as ELISA titers, was significantly higher, remaining up to 70 d after immunization.

Generation of an immune response to a protein or peptide antigen requires the cognate interaction between antigen-specific B and T cells (42). Vaccines designed for antibody-mediated protection must therefore contain both B- and T-cell epitopes. As the monoclonal antibody for the ELISA is specific for the T-binding site in the 18kDa-hsp, we concluded that its biologic activity was also preserved after being modified. A strik-

ing increase in the antibody production could be achieved in the presence of this small protein vehiculated into liposomes or conjugated into a supramolecular aggregate, therefore making it a proper carrier candidate. Furthermore, the scale-up design (20) and the economic study to run the production of the 18kDa-hsp (21) are ready. So, their future application as carrier systems for low antigenic recombinant proteins or peptides may become a reality and encourages further studies using this 18kDa-hsp as a carrier protein model system for use in animal or human vaccination.

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