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(54) Title: IMMUNOGENIC COMPOSITIONS CONTAINING ANTIGENS, GENE VECTORS AND ADJUVANTS-LOADED BIODEGRADABLE MICROSPHERES

(57) Abstract: The present invention relates to compositions which are able to stimulate an immune response in a host inoculated with an antigen of interest or the gene vector that codifies it. This composition includes microspheres based on copolymers derived from lactic acid glycolic acid encapsulating antigens, gene vectors and/or adjuvants with immunostimulatory properties obtained from mycobacterial fractions. The materialization of the present invention is the use of trehalose dimycolate associated to a heat shock protein or to the plasmid containing the gene that codifies it, encapsulated in microspheres with size smaller than 10 μm, capable of inducing specific antobodies and cellular immune responses, as well as the secretion of cytokines implicated in the protection against infectious diseases and cancer, following administration of a single dose or several doses.

"IMMUNOGENIC COMPOSITIONS CONTAINING ANTIGENS, GENE VECTORS AND ADJUVANTS IN BIODEGRADABLE MICROSPHERES"

The present invention is related to processes of production of pharmaceutical compositions which are able to stimulate a specific immune response, cellular and/or humoral, in a host immunized with an antigen or a gene vector that codifies the antigen of interest. Such compositions involve biodegradable microspheres based on a copolymer derived from polyesters, capable of encapsulating and controlling the release of antigens, gene vectors, and adjuvants that stimulate mediators of the immune response.

It is known that a class of compounds, including 6-mono and 6,6 diesters α,α-D-trehalose (among them the ester groups that contain 30 to 90 atoms of carbon, known as trehalose monomycolate and trehalose dimycolate, respectively), are able to promote recruitment of antigen presenting cells and cytokine induction, being therefore potential candidates as adjuvants in vaccines and immunotherapy. In the present invention, the term "adjuvant" refers to a substance that does not share immune epitopes with the antigen of interest itself, but is able to stimulate the immune response against the antigen of interest.

Patent US 4,340,588 describes the use of trehalose dimycolate as adjuvant in a vaccine composition containing

antigens of *Brucella abortus* (BA, 45/20 strain). In this composition, impure natural mixtures of trehalose dimycolate were obtained from several sources and were denominated "cord factor". These factors usually present 90% dimycolate and 10% monomycolate, as well as a small amount of exogenous material, which contains from 95 to 99% trehalose dimycolate after appropriate purification by chromatographic techniques.

The administration of vaccines containing antigen and trehalose dimycolate in an oil-in-water emulsion induced a significant reduction of the infection, when compared to a composition based only on soluble antigens of BA 45/20 strain. In this study the extract was mixed with oil as an emulsion. The inclusion of an oil phase is justified by its importance as a vehicle for extracts obtained from mycobacteria cellular wall, with the objective of stimulating an appropriate immune response, although it is known that the presence of this oil phase increases the occurrence of severe undesirable reactions in the administration site and can cause granuloma.

In an attempt to overcome the problems attributed to the presence of oil in preparations with immunogenic activity, the use of aqueous suspensions containing an insoluble fraction of mycobacteria cellular wall was proposed (US 5,759,554). Aqueous suspensions are prepared

by the following steps: (i) rupture of the bacteria and, subsequent centrifugation; (ii) elimination of protein from the fraction based on cellular wall (cellular debris obtained in the centrifugation step) employing proteolytic enzymes; (iii) treatment with detergents and washing of fraction resulting from step (ii), (iv) freeze-drying and (v) suspension of the freeze-dried fraction in an aqueous solution, such as saline solution. In that invention, the stimulation of the immune system through the employment of a suspension based on mycobacterial fractions stimulates the human or animal organism to neutralize an infectious agent or delay the growth of cancerous cells. However, this procedure does not allow a controlled adjuvant release, which could result in optimization of the immune response.

Processes that target the antigen, gene vectors and/or adjuvant have also been described in the literature, as in patent US 5,643,605. In this invention, the use of adjuvant and/or microencapsulated antigens is described for therapeutic or prophylactic purposes.

The composition presented under patent US 5,643,605 contains microspheres of PLGA (D-L-lactide-co-glycolide) with an average diameter varying from 20 to 100 μm encapsulating such immunoadjuvants. The adjuvant used was saponin (QS21) or muramildipeptide (MDP), which are able to stimulate an immune response where: (i) the volume of the

aqueous adjuvant incorporated into the polymer is equal or lower than 1 mL for 3 grams of polymer; (ii) the molar ratio between monomers of lactic and glycolic acid can vary from 100:0 to 0:100; (iii) the inherent viscosity of PLGA polymer varies from 0.1 to 1.2 dL/g; (iv) the diameters of the microspheres vary from 20 to 100 μm, and (v) the adjuvant is released from microspheres according to a triphasic model in which, during the first phase, less than 30% of the adjuvant is released in a period of 1 day; during the second phase, less than 10% of the adjuvant is released after a period of 1 day, which varies between approximately 30 and 200 days; and a third phase, where the remaining adjuvant is released after the precedent phases. However, it is important to point out that the diameter of the microspheres presented in this invention varies between 20 and 100 μm, hindering the induction of the immune response when used by administration routes such as oral or pulmonary. The ideal diameter for these routes should be comprised between 1 and 10 μm, which, as described in the literature, is also the ideal range for subcutaneous administration of immunogenic compositions. In this same invention, saponin was dissolved in ethanol solution and added to the polymeric phase and, in another formulation, MDP was dissolved in an aqueous phase that was added to the

polymeric phase. In both preparations, the phase containing the adjuvant corresponds to the inner aqueous phase of the preparation method. However, this procedure does not allow the addition of adjuvants that are soluble in non-polar solvents.

Several vaccine formulations use adjuvants with the purpose of optimizing the immune response when administered with the antigen, mainly in subunit vaccines. In the last decade, the progresses in vaccine development allowed the introduction of new strategies for obtaining and producing antigens, as well as new ways to administer and present those antigens to the immune system. These strategies allowed innovations, particularly in the context of the development of safer, effective and versatile vaccines. In this context, the DNA vaccines appeared as an interesting alternative to elicit protection, especially when cellular immune response is required.

Patent US 6,048,551 describes a method of preparation of microspheres containing gene vectors which are employed in the treatment of tumors. In this invention, the multiple emulsion method was used. The gene vector was dissolved in an aqueous phase and encapsulated, associated or not to antiviral drugs that were also dissolved in the aqueous phase. The diameter of the particles ranged between 1 and 200 μm.

Patent US 6,309,569 claims a method of encapsulating DNA in a polymer microparticle where the excess of organic solvent is extracted by adding the (water-in-oil)-in-water emulsion to an aqueous phase to extract the solvent, thereby forming polymer microparticles of a size up to 10 μm in diameter, containing DNA in aqueous solution.

So far, a method that allows the coencapsulation of an antigen or a gene vector and an immunoadjuvant in the same preparation of PLGA-derived biodegradable microspheres is yet to be described. Therefore, in spite of the efforts that have been made in the sense of offering controlled delivery formulations, containing or not a co-adjuvant molecule which is able to improve immune response, the state of the art still lacks a passive phagocytic cell targeting system based on microspheres, with an appropriate particle diameter so that it could be administered through different routes and still target specific cellular populations.

An important reason for the development of such system refers to the need of an effective vaccine against tuberculosis.

This invention presents an immunogenic composition involving, at least, adjuvant and/or protein; and/or plasmids containing protein-expressing genes, entrapped into polymeric microspheres with an average diameter

smaller than 20 μm, preferentially between 1 and 10 μm. Therefore, the composition contains, besides the adjuvant, an antigen or a gene vector that codifies the antigen (preferentially from parasites and pathogenic agents), which include antigens of extra and intracellular bacteria, eucariotic organisms such as fungi and protozoa, among others. The adjuvants are, preferentially, those which stimulate citokyne production. Ideally, the plasmid carrying the gene of Mycobacterium proteins as, for instance, the hsp65 gene (heat shock protein of 65 kDa from M. leprae), or still, the purified or recombinant protein may be encapsulated in polymeric microspheres derived from lactic acid and copolymers of lactic and glycolic acid (monomer ratio varying between 100:0 and 0:100). Moreover, the trehalose dimycolate may be encapsulated in microspheres of PLGA. The system which includes trehalose dimycolate and/or the plasmid containing the gene of hsp65 protein, or still the protein itself, can also be applied in the prevention and therapy of tuberculosis, when the disease is already installed in the human or animal host.

The microencapsulation of a gene vector, antigen and/or adjuvant according to the present invention can be obtained by the following protocols:

Protocol A:(i) dissolution of the polymer derived from lactic and glycolic acids (ratio in % of weight between

100:0 and 0:100) in an organic solvent, which is non miscible or partially miscible in water in a concentration between 0.2 and 10%, as for example, ethyl acetate, methylene chloride, chloroform, among others; (ii) dissolution of the lipophilic antigen and/or adjuvant in the solution (i), that contains the polymer, (iii) addition of the solution obtained in step(ii) to a polyol solution, for example, polyvinyl alcohol (1 to 10%), aiming the formation of an oil-in-water emulsion, (iv) stirring of this emulsion under an appropriated speed (200 to 1000 rpm), in order to obtain microspheres with an average diameter smaller than 10 μm, preferentially between 1 and 5 μm, and (v) solvent elimination under agitation, which can be done by using co-solvents, by vacuum evaporation or other techniques that allow solvent removal, not compromising the stability of the system. The suspension of microspheres obtained may be, alternatively, collected by centrifugation or filtration, washed with an aqueous solvent, then freeze-dried.

Protocol B: (i) dissolution of the polymer derived from lactic and glycolic acids (ratio in % of weight between 100:0 and 0:100) in an organic solvent in a concentration between 0.2 and 10%, for example, methylene chloride, ethyl acetate, chloroform, among others, with or

without a lipophilic adjuvant such as trehalose dimycolate;(ii) dissolution of the gene vector, antigen and/or adjuvant of hydrophilic nature, in 0.1 to 1 mL of an aqueous phase, (iii) addition of the solution obtained in (ii) to the polymeric solution obtained in (i), followed by agitation under a speed ranging between 500 to 15,000 rpm, leading to the formation of a water-in-oil emulsion (or dispersion), (iv) addition of this primary emulsion (or dispersion), after adequate period of agitation (1 to 10 minutes), to an emulsifying aqueous phase, typically composed by a polyol solution, for example, polyvinyl alcohol (1 to 10%), aiming the formation of a water-in-oil-in-water emulsion; (v) agitation of the multiple emulsion obtained in (iv) under appropriated speed in order to remove the organic solvent (100 a 1000 rpm), resulting in microspheres with an average diameter smaller than 10 μm, preferentially between 1 to 5 μm, which may be collected by filtration or centrifugation, washed with an aqueous solvent and finally freeze-dried.

It is important to mention that other methods known by the state of the art could be employed to obtain antigens and/or adjuvants-loaded microspheres. This is the goal of this invention, which is not limited by the techniques presented herein.

The present invention is described in details by the following examples:

EXAMPLE 1 - Preparation of trehalose dimycolate

One hundred grams of *Mycobacterium tuberculosis* H37Rv strain are homogenized several times in a chloroform-methanol mixture (1:1, vol/vol). The extracted material (18 g) is fractionated as previously described for trehalose dimycolate purification (Silva, C. L., S. M. Ekizlerian, and R. A. Fazioli. 1985. Role of cord factor in the modulation of infection caused by *Mycobacteria*. Am. J. Pathol. 118:238-247). The purified glycolipid (0.530 g) migrates as a unique band when submitted to thin-layer chromatography, similarly to the commercial product (SIGMA, St. Louis, USA).

EXAMPLE 2 - Preparation and characterization of microspheres containing trehalose dimycolate

Microspheres are prepared following the emulsion and solvent evaporation technique (Lewis, D. H. 1990. Controlled release of bioactive agents from lactide/glicolide polymers. In Chasin M & Langer R. Biodegradable polymers as drug delivery systems. Marcel Dekker, New York, 1-43). Five mg of trehalose dimycolate (SIGMA) and 125 mg of PLGA (50:50)biodegradable (Resomer RG 505, MW 78,000, Boehringer Ingelheim, Germany) were diluted in 30 mL of methylene chloride. This organic phase was

mixed with 100 mL of an aqueous phase containing 3% of polyvinyl alcohol (Mowiol® 40-88, SIGMA Aldrich Chemicals) as surfactant, aiming the formation of an oil-in-water stable emulsion. In order to allow organic solvent evaporation, a mechanical agitation (800 rpm) was applied for a 6-hour period at room temperature. Microspheres were collected by centrifugation at 10.000 x *g*, washed 3 times with sterile water or saline, freeze-dried and stored at 4°C. Presence of trehalose dimycolate in microspheres was determined by thin-layer chromatography after dissolution of the particles in methylene chloride. Diameter of the particles was determined by laser difractometry in a CILAS 1064 Liquid apparatus (Cilas, France) showing average diameter between 1 and 5 μm. Results were expressed as cumulative values of % under determined diameter. Unloaded PLGA microspheres or microspheres containing trehalose dibehenate (Sigma, St. Louis, USA) or control lipid as unrelated control (a glycerides mixture, Sigma, St. Louis, USA) are prepared by the same procedure and used as controls for experimental protocols.

EXAMPLE 3 - Preparation of plasmids

E. coli, DH5α strain, transformed with the gene vectors pCDNA3 or pCDNA3modified (pCDNA3m), and with the plasmids containing the hsp65 gene (pCDNA3-hsp65

pCDNA3modified-hsp65) are cultured in LB BROTH BASE medium (GIBCO BRL, Scotland) containing 100 μg/mL of ampicillin (SIGMA). The construct pCDNA3modified-hsp65 is derived from pCDNA3 vector (Invitrogen®, Carlsbad, CA, USA), which is previously digested with Bam HI and Not I (Gibco BRL, Gaithersburg, MD, USA) and then, a fragment of 3.3 kb corresponding to the *M. leprae* hsp65 gene and the CMV intron A (citomegalovirus) is inserted in the vector. The vector without the hsp65 gene is used as control. Plasmids are purified by anion exchange resin (Concert High Purity Maxiprep System, GIBCO BRL). Evaluation of plasmid concentration is carried out by spectrophotometry in λ = 260 and 280 nm using a Gene Quant II apparatus (Pharmacia Biotech).

EXAMPLE 4 -Preparation of recombinant protein

E. coli, BL21 strain, transformed with pIL-161 plasmid containing the *M. leprae* hsp65 gene, are cultured in 500 mL of LB BROTH BASE liquid medium (GIBCO BRL, Scotland) containing ampicillin (SIGMA) in the concentration of 100 μg/mL and IPTG (Isopropylthio-β-D-Galactoside, GIBCO BRL) 0.1 M, at 37°C, under agitation at 200 rpm in an incubator shaker for 18 hours. After incubation, cells are collected by centrifugation at 7,500 rpm for 15 minutes in a J2HS centrifuge (Beckman, rotor JS-7.5), ressuspended in 10 mL

of CE buffer (30 mM Sodium citrate, 10 mM EDTA, pH 6.1) and disrupted by means of 3 pulses of 1 minute and 100 W, using a Vibra Cell™ ultrasound probe (Sonics & Materials, USA) in ice bath. After a 16,500 rpm centrifugation for 20 minutes, the supernatant is discharged and the pellet is ressuspended in 30 mL of UPE buffer (6 M urea, 20 mM EDTA, 50 mM monobasic potassium phosphate, pH 7.0) and vortexed for 3 minutes. Suspension is kept under gentle agitation at room temperature for 15 minutes and then centrifuged at 16,500 rpm for 20 minutes. Ammonium sulfate is added to the supernatant to a final concentration of 1 M and incubated in ice bath for 30 minutes. After centrifugation at 16,500 rpm for 20 minutes, the pellet is ressuspended in 3 mL of PBS and exhaustively dialyzed against PBS.

EXAMPLE 5 - Preparation of Plasmid or recombinant protein-loaded microspheres

Microspheres are obtained by multiple emulsion followed by solvent evaporation method: the aqueous phase (0.3 mL) containing only the plasmid vector that does not codify the hsp65 protein (pCDNA3 or PcDNA3modified); or the vector containing the gene that codifies the hsp65 protein (pCDNA3-hsp65, pCDNA3modified-hsp65); or only the recombinant hsp65 protein is emulsified in 30 mL of methylene chloride containing 400 mg of PLGA 50:50 (Resomer RG 505 from Boehringer Ingelheim), under strong agitation

(8000 rpm) using an Ultraturrax T50 homogenizer (IKA® - Labortechnik, Germany), in order to obtain a water-in-oil primary emulsion. For preparation of microspheres containing plasmid, 4 mg of plasmid are dissolved into 0.3 mL of aqueous phase and, in case of recombinant hsp65, 1 mg of protein is dissolved into 0.3 mL of aqueous phase. This emulsion is added to an external aqueous phase (100 mL), containing 3% of polyvinyl alcohol (Mowiol® 40-88, Aldrich Chemicals) as surfactant, and homogenized to form a multiple water-in-oil-in-water emulsion. The organic solvent is removed by evaporation at room temperature under agitation (300 to 800 rpm) using a Eurostar homogenizer (IKA® - Labortechnik, Germany) for 6 to 10 hours. Microspheres are collected by centrifugation at 10.000 x *g* in a Himac CR21 centrifuge (Hitachi, rotor R20A2), washed 3 times with sterile water, freeze-dried, then stored at 4°C. Particle diameter is evaluated by laser difractometry in a CILAS 1064 Liquid apparatus (Cilas, France). The average particle diameter should be comprised between 1 and 5 μm.

EXAMPLE 6 - Preparation of dimycolate and plasmid or dimycolate and protein-loaded microspheres

Microspheres are obtained by multiple emulsion and solvent evaporation method: the aqueous phase (0.3 mL) containing pCDNA3, PcDNA3modified, pCDNA3-hsp65,

PcDNA3modified-hsp65 or recombinant hsp65 (4 mg for plasmid or 1 mg for protein), is emulsified in 30 mL of methylene chloride containing 0.5 mg of trehalose dimycolate and 400 mg of PLGA 50:50 (Resomer RG 505 da Boehringer Ingelheim) under strong agitation (8,000 rpm), using an Ultraturrax T50 homogenizer (IKA® - Labortechnik, Germany), in order to obtain a water-in-oil primary emulsion. This emulsion is added to an external aqueous phase (100 mL), containing 3% of polyvinyl alcohol (Mowiol® 40-88, Aldrich Chemicals) as surfactant, and homogenized to form a multiple water-in-oil-in-water emulsion. The organic solvent is removed by evaporation at room temperature under agitation (300 to 800 rpm), using a Eurostar homogenizer (IKA® - Labortechnik, Germany) for 6 hours. Microspheres are collected by centrifugation at 10.000 x *g* in a Himac CR21 centrifuge (Hitachi, rotor R20A2), washed 3 times with sterile water, freeze-dried and stored at 4°C. Particle diameter is evaluated by laser difractometry in a CILAS 1064 Liquid apparatus (Cilas, France). The average particle diameter should be between 1 and 5 μm.

EXAMPLE 7 - Evaluation of hsp65 expression in eucariotic cells transfected with plasmid-loaded microspheres

J774 cells (tumoral lineage cells originally from BALB/c mice) and J774-hsp65 (J774 cells transfected with retroviral vector [pZIPNeoSV(x)] carrying the *M. Leprae* hsp65 gene) are cultured in 1640 RPMI medium (SIGMA) supplemented with 2 % of heat inactivated fetal calf serum, at 37 °C in a 5 % CO_2 atmosphere. One hundred µL of cell suspension ($5x10^5$ cells) are placed on sterile cover slips in a 24 well plate and incubated for 3 hours to allow cell adhesion. After incubation, 1 mL of RPMI medium, is added to each sample and treated as follows:

A) J774 cells receive 1 mL of RPMI medium and are used as negative control for hsp65 expression;

B) J774-hsp65 cells receive 1 mL of RPMI medium and are used as positive control for hsp65 expression;

C) J774 cells receive 1 mL of RPMI medium containing pCDNA3modified-hsp65-loaded microspheres (10 microspheres/cell). Particles are ressuspended in medium and the concentration of this suspension is adjusted to $5x10^6$ particles/mL, counting the particles in a Neubauer chamber;

D) J774 cells receive 1 mL of RPMI medium containing pCDNA3modified-hsp65 complexed with Lipofectin® (GIBCO BRL).

Twenty-four hours after incubation, the culture supernatants are removed and replaced with 1 mL of RPMI medium. Medium is substituted every 48 hours. Immunocytochemistry analyses are performed 10 and 20 days after the beginning of the test.

For the immunocytochemistry evaluation, the cells are washed 3 times in PBS solution containing 1% of bovine serum albumin (BSA) (SIGMA). They are fixed in 4% paraformaldehyde solution for 30 minutes at room temperature and then rewashed . All the washes are performed 3 times. After that, endogenous peroxidase blockage is carried out by incubation with PBS containing 3% of H_2O_2 for 30 minutes at room temperature. After washing, the cells are incubated with blocking buffer (3 % of rabbit serum, 1% of BSA and 0.01% of Triton X 100) for 1 hour at room temperature, in order to block nonspecific linkages. Following that, anti-hsp65 antibody is added and cells are incubated in humid chamber at 4°C overnight. The monoclonal antibody is diluted (1:100) in blocking buffer and, after incubation and washing, samples are incubated with biotinilated anti-mouse IgG antibody (B-7022, Sigma), diluted (1:200) in blocking buffer for 1 hour, at room temperature. After washing, samples are further incubated for 30 minutes at room temperature with streptavidin biotin-peroxidase complex (Streptoavidin-Dako Corporation,

CA-USA). They are then washed and revealed with 3-3'-Diaminobenzidine substrate (SIGMA) (5 mg in 10 ml of PBS), adding 180 µL of H_2O_2 (20 volumes). The reaction is stopped with distilled water and the cells are stained with Mayer hematoxilin for 5 to 10 minutes at room temperature. After washing with distilled water, they are treated with 0.5% ammonium hydroxide solution. Following that, they are again washed, dried at room temperature, and placed on slides with Canadian balsam. Negative control is obtained by replacing primary antibodies for PBS or irrelevant antibodies with same isotypes. The cells are visualized and pictures are taken using an Aristoplan microscope (Leitz), equipped with the MC80DX photography system. It was previously demonstrated that the encapsulating process does not affect the DNA functionality, since macrophages submitted to the procedure describe above are able to express the hsp65 protein.

EXAMPLE 8 - Immunization and challenge infection

BALB/c mice, 6 to 8 weeks old, were obtained from the animal facilities at the School of Medicine of Ribeirão Preto, University of São Paulo, and maintained in standard laboratory conditions. All procedures were carried out according to the Ethic Committee recommendations.

Mice were immunized with different microsphere formulations by intramuscular or intratracheal route.

Microsphere doses varied from 100 to 500 mg/kg and the schedules were of single dose for intratracheal route and 1 or 3 doses for intramuscular route. Mice were either challenged or killed, 30 or 60 days after administration of the microspheres, and the immune response was evaluated.

As challenge infection, mice received, by intratracheal route, a dose of 10^5 colony-forming units (CFU) of *M. tuberculosis* H37Rv strain, under anesthesia with 200 µl of a 2.5% of tribromoethanol (Sigma) solution in PBS. Infected animals were kept under suitable biosafety conditions. Infected animals were killed in different time intervals in order to characterize inflammatory reaction in lungs and evaluate protection.

EXAMPLE 9 - Cytokine measurement

Cytokine levels produced by lung and spleen cells from mice immunized with microspheres are measured by ELISA. Total lung tissue is homogenized for 5 minutes in an Ultraturrax T 25 IKA (Labortechnik, Germany) at 4°C. Homogenized tissue is centrifuged at 10,000 x *g* for 15 minutes and the supernatant is filtered through a 0.22 µm membrane, and used to measure cytokine production. Spleen cells are cultured at 37°C in 5% CO_2 atmosphere and stimulated in vitro with concanavalin A, as nonspecific stimulus, or with recombinant hsp65 as specific stimulus.

Forty-eight hours later, the culture supernatant is collected for cytokine analysis. Capture and biotinilated monoclonal antibodies specific for TNF-α (MP6-XT22, MP6-XT3), IL-10 (JES5-2A5, JES5-16E3), IL-6 (MP5-20F3, MP5-32C11), IL-4 (BVD4-1D11, BVD6-24G2), IFN-γ (R4-6A2, XMG1.2) and IL-12 (C15.6, C17.8), as well as recombinant cytokines, may be purchased from Pharmingen (San Diego, CA). Levels of TNF-α, IL-10, IL-6, IL-4, IFN-γ and IL-12 are determined in supernatant by ELISA technique, as previously described (Haagmans, B. L., A. J. van den Eertwegh, E. Claassen, M. C. Horzinek, and V. E. Schijns. 1994. Tumor necrosis factor-alpha production during cytomegalovirus infection in immunosuppressed rats. J. Gen. Virol. 75:779-787), according to manufacturer instructions (Pharmingen, San Diego, CA). For each assay, a standard curve is built with recombinant mouse rTNF-α, rIL-10, rIL-6, rIL-4, rIFN-γ, or rIL-12. The limit of detection for all evaluated cytokines is 15 pg/mL.

EXAMPLE 10 - Nitric oxide measurement

Cells from bronchoalveolar lavage recovered from mice injected with microspheres containing trehalose dimycolate are cultured in RPMI medium containing 10% of fetal calf serum (GIBCO-BRL, Grand Island, NY), 10 mM Hepes, 20 mM sodium bicarbonate, penicillin, and streptomycin at 100

μg/ml (GIBCO-BRL). Production of nitric oxide is evaluated by measuring nitrite (NO_2^-) production in cell culture supernatant by Greiss method (Stuehr, D.J., and C. F. Nathan. 1989. Nitric oxide. A macrophage product responsible for cystostasis and respiratory inhibition in tumor target cells. J. Exp. Med. 169:1543-1555). The standard curve is constructed using a serial dilution of a 200 μM sodium nitrite solution. The limit of detection is 3 μM per 10^5 cells.

EXAMPLE 11 - Evaluation of protection against challenge infection

In order to evaluate the protection conferred by the different formulations against challenge infection with *M. Tuberculosis* H37Rv strain, the animals previously immunized and challenged (as described in EXAMPLE 8) are killed. Lungs from these animals are removed, weighed, and homogenized. Serial dilutions of the lung homogenate are plated on 7H11 medium. After 21 days of incubation at 37°, the CFU number in each plate is counted.

In all experiments, the formulations which are object of the present invention presented suitable characteristics, such as mean diameter smaller than 10 μm (more precisely between 1 and 5 μm), therefore being easily taken up by phagocytic cells, allowing passive targeting of

the encapsulated material. The process of encapsulation does not affect the DNA functionality, since macrophages treated with pCDNA3modified-hsp65-loaded microspheres are able to express the protein.

The present invention is further explained by the annexed tables. Table I and II show that recombinant hsp65-loaded microspheres are able to elicit specific immune response in both local an systemic levels after intratracheal administration. This response was evaluated by measurement of interferon-γ in spleen cells culture supernatant (Table I) and by measurement of IL-12 levels in lung homogenate (Table II) from immunized BALB/c mice. Interferon-gamma (IFN-γ) levels were significant after *in vitro* stimulation with recombinant hsp65 in mice immunized with recombinant hsp65-loaded microspheres. Mice immunized with recombinant hsp65 (r-hsp65) in PBS or unloaded microspheres did not produce significant levels of IFN-γ. IFN-γ levels produced after *in vitro* stimulation with concanavalin A (ConA) were used as positive control. Interleukin 12 (IL-12) levels were significant in mice which received r-hsp65 in PBS or loaded into microspheres. This result confirms the possibility of administering the formulations resulting from the present invention by a route other than the parenteral one.

TABLE I: IFN-γ production (pg/mL) in spleen cells culture supernatant from immunized BALB/c mice, 30 days after immunization

Formulations used in immunization	Stimuli		
	Medium	ConA	Hsp65
r-hsp65 in PBS	140.4 (±184.45)	7,264.9(± 274.8)	470(± 32.1)
r-hsp65 into microspheres	64.7 (±11.9)	5,274.8(± 628.3)	1,036.6(± 93.1)
Unloaded Microspheres	157.3 (±123.9)	8,276.1(± 482.7)	336.7(± 47.3)

TABLE II: IL-12 production (pg/g) in lungs homogenate from immunized BALB/c mice, 30 days after immunization

Formulations used in immunization	IL-12 (pg/g of lung)
r-hsp65 in PBS	17,360.62 (± 893.4)
r-hsp65 into microspheres	17,827.36 (± 978.6)
Unloaded microspheres	6,506.60 (± 843.4)

However, after parenteral administration, the formulations object of the present invention were also able to elicit specific immune response. BALB/c mice were immunized with a single dose of 5 mg of microspheres containing plasmid/mouse by intramuscular route. Sixty days after immunization, there was evidence of specific immune response, demonstrated by production of IFN-γ, IL-12, and IL-6 in spleen cells culture supernatant As shown in Tables III, IV and V, this response was achieved with a reduced dose of plasmid compared to conventional vaccination protocol, in which DNA is administered as a solution in three separated doses , as described in invention patent WO95/31216 and in studies from Silva and co-workers (Lowrie et al., Nature 400:269-271, 1999). A single dose of 5mg of microspheres (containing 30 μg of plasmid) was able to elicit an immune response similar to that elicited by

intramuscular injection of three doses of 100 μg of naked plasmid. This result demonstrates the advance attained by this invention, in regard to reduction of the amount of DNA necessary to elicit a significant immune response, thus reducing the number of injections required.

TABLE III: IFN-γ production (ng/mL) in spleen cells supernatant from BALB/c mice immunized by intramuscular injection with plasmid or r-hsp65 encapsulated into microspheres , 60 days after immunization

Formulations used in immunization	Stimuli		
	Medium	ConA	hsp65
pCDNA3m into microspheres	2.94(±0.82)	125.07(±7.78)	3.71(±1.14)
pCDNA3m-hsp65 into microspheres	2.41(±0.31)	239.92(±12.79)	24.95(± 1.24)
r-hsp65 into microspheres	2.75(±0.15)	138.47(±13.15)	5.21(±0.28)

Table IV: IL-12 production (pg/mL) in spleen cells culture supernatant from BALB/c mice immunized by intramuscular injection with plasmid and r-hsp65 loaded into microspheres, 60 days after immunization

Formulations used in immunization	Stimuli	
	Medium	hsp65
pCDNA3m into microspheres	699.67 (±83.15)	735.67 (±217.68)
pCDNA3m-hsp65 into microspheres	1,053.30 (±234.42)	1,920.67 (± 320.20)
r-hsp65 into microspheres	719.67 (±129.31)	856.01 (±44.23)

Table V: IL-6 production (pg/mL) in spleen cells culture supernatant from BALB/c mice immunized by intramuscular injection with plasmid and r-hsp65 encapsulated into microspheres , 60 days after immunization

Formulations used in immunization	Stimuli		
	Medium	ConA	hsp65
pCDNA3m into microspheres	364.83 (±25.7)	5,242.79 (±705.4)	1,816.03 (±1,490.5)
pCDNA3m-hsp65 into microspheres	565.67 (±83.8)	4,309.89 (±359.1)	3,730.44 (± 703.5)
r-hsp65 into microspheres	494.40 (±148.4)	5,178.01 (±815.7)	7,436.17 (± 281.0)

Concerning immunostimulatory properties of TDM-loaded microspheres, Tables VI and VII show that, after

intratracheal administration, the formulation obtained in this invention was able to elicit the production of several cytokines by lung cells, as well as the activation of alveolar macrophages as determined by nitric oxide production. Table VI illustrates the results of cytokine production by lung cells from mice injected with microspheres containing TDM and controls (PBS, control lipid CtLip and trehalose dibehenate DBT). The animals were injected by intratracheal route. Sixty days later, their lungs were removed and homogenized for measurement of cytokine in supernatant by ELISA. Data are representative of one experiment, repeated 3 times.

Table VI: Cytokine production in lung homogenate from BALB/c mice treated by intratracheal route, 60 days after injection

Treatment	Cytokines (pg/mL)					
	IFN-γ	IL-12	IL-10	IL-6	IL-4	TNF-α
PBS	9,720 (±841)	13,125 (±2,203)	22,835 (±10,801)	7,783 (±1,412)	49,684 (±22,788)	2,804 (±418)
Control lipid into microspheres	8,920 (±421)	11,880 (±346)	23,935 (±2,744)	9,624 (±707)	43,342 (±5,764)	3,548 (±724)
DBT into microspheres	9,680 (±950)	15,915 (±2,034)	27,591 (±4,138)	11,985 (±1,758)	60,688 (±13,261)	3,659 (±412)
TDM into microspheres	19,200 (±4,111)	26,577 (±2,361)	47,163 (±3,707)	17,781 (±2,726)	111,504 (±1,786)	5,855 (±287)

Table VII illustrates the results of nitric oxide production by cells from bronchoalveolar lavage from mice

injected with microspheres containing TDM and controls (PBS, control lipid, and trehalose dibehenate). Around 10^5 cells from bronchoalveolar lavage were obtained in each experiment and cultured for 24 hours at 37°C in 5% CO_2 atmosphere. Following that, culture supernatant was collected and the nitrite concentration was measured by Greiss method. Statistical analysis was carried out by multiple comparison Dunnett's test and showed significant differences between treatment with TDM (* $p<0.01$) and DBT(*$p<0.01$), compared to PBS injected mice.

Table VII: Evaluation of nitric oxide levels in broncoalveolar lavage cells culture supernatant from BALB/c mice treated by intratracheal route.

Treatment	Concentration (μM) of NO_2 per 10^5 cells
PBS	0.455 (±0.63)
Unloaded microspheres	0.045 (±0.05)
Control lipid into microspheres	0.045 (±0.05)
DBT into microspheres	6.5 (±0.28)
TDM into microspheres	8.6 (±1.41)

Table VIII illustrates the IFN-γ levels produced in spleen cell culture supernatant from BALB/c mice immunized

with microspheres containing plasmid plus TDM and controls

Table VIII: IFN-γ production (pg/mL) in spleen cells culture supernatant from BALB/c mice immunized, 30 days after immunization

Formulations used in immunization	Stimuli		
	Medium	ConA	hsp65
Nonimmunized	856.2 (±86.3)	26,761.1 (±3,840.3)	959.8 (±50.0)
TDM into microspheres	1,204.1 (±264.6)	14,043.2 (±3,541.4)	1,153.4 (±428.8)
pCDNA3m and TDM into microspheres	1,010.5 (±141.2)	21,886.6 (±11,395.6)	1,644.1 (±576.3)
pCDNA3m-hsp65 and TDM into microspheres	1,077.4 (±373.5)	14,711.3 (±5,129.8)	18,806.4 (±1,057.4)

Table IX illustrates the levels of antibodies in serum from BALB/c mice immunized with microspheres containing plasmid plus TDM and controls, 30 days after immunization.

TABLE IX: Evaluation of antibody production in serum from BALB/c mice immunized by intramuscular injection of microspheres containing plasmid plus TDM, 30 days after immunization

Formulations used in immunization	Title (Log 2)		
	IgG	IgG1	IgG2a
Nonimmunized	1.90(± 0.13)	0.64(± 0.04)	0.91(± 0.22)
Unloaded microspheres	2.56(± 0.17)	0.84(± 0.04)	1.63(± 0.19)
TDM into microspheres	2.25(± 0.34)	1.11(± 0.35)	1.21(± 0.03)
PCDNA3m plus TDM into microspheres	3.23(± 0.39)	2.99(± 0.47)	2.64(± 0.14)
pCDNA3m-hsp65 plus TDM into microspheres	8.95(± 0.10)	2.61(± 0.07)	10.15(± 0.04)

Tables X, XI and XII illustrate cytokine levels produced in lung homogenate from BALB/c mice challenged with *M. tuberculosis* H37Rv strain after injection of microspheres containing plasmid plus TDM and controls.

Table X: IL-10 production (pg/mL) in lung homogenate from BALB/c mice immunized and challenged with *M. tuberculosis*

Formulations used in immunization	IL-10 (pg/g of lung)
Nonimmunized	4,799.15 (± 1,503.7)
Unloaded microspheres	3,525.32 (± 2,594.4)
TDM into microspheres	2,428.69 (± 706.8)
pCDNA3m and TDM into microspheres	4.654.18 (± 300.9)
pCDNA3m-hsp65 and TDM into microspheres	14,250.30 (± 6,580.9)

Table XI: IL-12 production (pg/mL) in lung homogenate from BALB/c mice immunized and challenged with *M. tuberculosis*

Formulations used in immunization	IL-12 (pg/g of lung)
Nonimmunized	25,817.28 (± 3,286.2)
Unloaded microspheres	26,945.16 (± 2,346.0)
TDM into microspheres	15,852.67 (± 1,002.4)
pCDNA3m and TDM into microspheres	27,269.74 (± 1,610.9)
pCDNA3m-hsp65 and TDM into microspheres	36,825.70 (± 662.0)

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Table XII: IFN-γ production (pg/mL) in lung homogenate from BALB/c mice immunized and challenged with *M. tuberculosis*

Formulations used in immunization	IFN-γ (pg/g of lung)
Nonimmunized	2,711.34 (± 1,225.8)
Unloaded microspheres	2,422.64 (± 2,077.9)
TDM into microspheres	1,728.51 (± 363.9)
pCDNA3m and TDM into microspheres	1,411.67 (± 758.9)
pCDNA3m-hsp65 and TDM into microspheres	7,775.13 (± 2,855.2)

Table XIII illustrates the ability of different microsphere formulations containing DNA or protein plus TDM, to confer protection in BALB/c mice against challenge with virulent strain of *M. Tuberculosis*.

Formulations used in immunization	CFU number/g of lung(log 10)
Nonimmunized	6.32 (± 0.033)
Unloaded microspheres	5.27 (± 0.048)
TDM into microspheres	6.84 (± 0.380)
PCDNA3m plus TDM into microspheres	3.85 (± 0.278)
pCDNA3m-hsp65 plus TDM into microspheres	6.98 (± 0.023)

As shown in Table XIII, only the formulation containing pCDNA3m-hsp65 plus TDM loaded into microspheres was able to protect mice from infection. It is important to

point out that a single administration, containing a dose 10-fold lower of plasmid was required to induce protection similar to that obtained by naked DNA protocol. The optimal encapsulation rate of protein was up to 95%; and varied from 60 to 70% for plasmid. The optimal adjuvant/polymer rate was 6 μg of adjuvant to 1 mg of polymer.

CLAIMS:

1 - A process for obtaining immunogenic compositions containing an antigen or a gene vector and an imunoadjuvant co-entrapped into biodegradable microspheres which are able to elicit a specific immune response, including cellular and humoral response, after administration of a single dose or several doses.

2 - A pharmaceutical composition containing an antigen or a gene vector plus an immunoadjuvant, as described in claim 1, which can be administered through upper airway (intratracheal, pulmonary and nasal), oral and parenteral routes.

3 - A pharmaceutical composition as described in claims 1 and 2, containing antigens or gene vectors plus immunoadjuvant, including polymers derived from lactic acid and copolymers derived from lactic and glycolic acids.

4 - A pharmaceutical composition as described in claims 1 and 2, containing antigens or gene vectors plus immunoadjuvants entrapped into microspheres that present an average diameter ranging from 1 to 20 □m.

5 - A pharmaceutical composition as described in claims 1 and 2, containing antigens or gene vectors plus immunoadjuvants where the process of production, as well as the organic solvents applied to it, do not induce lack of

functionality of the encapsulated antigen, plasmid, or immunoadjuvant.

6 - A pharmaceutical composition as described in claims 1 and 2, where the immunoadjuvant potentiates the immune response to the antigen or gene vector, reducing the required dose of antigen or gene vector capable of inducing immune response.

7 - A pharmaceutical composition as described in claims 1 and 2, where the immunoadjuvant potentiates the immune response to the antigen or gene vector, capable of eliciting both cellular and humoral specific immune responses after a single dose administration.

8 - A process for obtaining the composition described in claims 1 and 2, where in the first step of the process, the antigen or gene vector is added to the aqueous phase from the primary emulsion and the immunoadjuvant is added to the organic phase (which contains the polymer) of the same emulsion, forming a water-in-oil emulsion containing antigen or gene vector plus adjuvant.

9 - A process for obtaining the composition described in claims 1 and 2, where the water-in-oil emulsion obtained in claim 8 is re-emulsified in an aqueous phase containing an appropriate surfactant to obtain a water-in-oil-in-water emulsion.

10 – A pharmaceutical composition as described in claims 1 and 2, where the antigen is a recombinant or purified 65 kDa heat shock protein from *Mycobacterium leprae*.

11 - A pharmaceutical composition as described in claims 1 and 2, where the gene vector is the plasmid containing the gene which codifies the 65 kDa heat shock protein from *Mycobacterium leprae*.

12 - A pharmaceutical composition as described in claims 1, 2, and 7, where the adjuvant is the trehalose dimycolate.

13 - A pharmaceutical composition as described in claims 1, 2, and 7 where the antigen is the 65 kDa heat shock protein from *Mycobacterium leprae* and the adjuvant is the trehalose dimycolate.

14 - A pharmaceutical composition as described in claims 1, 2, and 7 where the gene vector is the plasmid containing the gene that codifies the 65 kDa heat shock protein from *Mycobacterium leprae* and the adjuvant is the trehalose dimycolate.

15 - A pharmaceutical composition as described in claims 1, 2, and 7, which following pulmonary administration is able to elicit an immune response characterized by:

- stimulation of cytokines production, such as IL-4, IL-6, IL-10, IL-12, IFN-□, and TNF-□;
- production of antibodies of IgG1 and IgG2a isotypes.

- increase of nitric oxide production by alveolar macrophages;
- stimulation of local and systemic immune response.

16 - A pharmaceutical composition as described in claims 1, 2, and 7, which following parenteral administration, is able to elicit an immune response characterized by:

- stimulation of cytokines production, such as IL-6, IL-10, IL-12, and IFN-□;
- production of serum immunoglobulins of Th1 pattern, which are specific for the antigen;
- production of antibodies of IgG1 and IgG2a isotypes, especially IgG2a isotype.

17 - Use of the composition described in claims 1, 2 and 7 to prevent infectious diseases which require a specific immune response characterized in claims 15 and 16, in humans and animal hosts.

18 - Use of the composition described in claims 1, 2, and 7 to treat, acting as therapeutic agent, infectious diseases, asthma, autoimmune diseases and cancer, which require a specific immune response characterized by claims 15 and 16, in humans and animal hosts.

19 - Use of composition described in claims 1, 2, and 7 to prevent tuberculosis in humans and animals;

20 - Use of the composition described in claims 1, 2, and 7 to treat tuberculosis in humans and animals.