REPORT

Liposomal lipids as immunoadjuvants for recombinant human Epidermal Growth Factor (rhEGF) and the main allergens (maDer s) of the Dermatophagoides siboney *dust mite*

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ABSTRACT

We studied the influence of lipid composition on the immunomodulatory capacity of liposomal vesicles. Liposomes composed of different phosphatidylcholines (PC) and cholesterol were assayed as adjuvants for rhEGF conjugated or not to P64k from *Neisseria meningitidis,* and dipalmitoylphosphatidylcholine (DPPC) liposomes with or without cholesterol were tested for the encapsulation of allergens (maDer s) and sticholysin II (StII), a pore-forming protein from *Stichodactyla helianthus*. The immunization of mice under these conditions produced high antibody titers against rhEGF even in the absence of P64k, composed of IgG2a and IgG2b subclasses, a delayed hypersensitivity response, lymphoproliferation, and increased survival rates in mice challenged with Ehrlich's ascitic tumor. This immune response was superior when employing saturated phosphatidylcholine liposomes, as compared to unsaturated liposomes (soy bean: sPC) or Alum. Additionally, the humoral allergen-specific immune response depended on the liposomal lipid composition and on the presence of StII. No allergic responses were detected in mice immunized with cholesterol-containing liposomes; and these preparations induced the highest IgG2a/IgG1 ratio, suggesting the presence of a Th1 response pattern. These findings constitute the first reported evidence on immunomodulatory effects of StII.

Introduction

The immunomodulatory properties of liposomes were first discovered by Allison and Gregoriadis in 1974 and later confirmed for a wide variety of antigens from bacteria, viruses, protozoa, tumors and other sources [1]. However, a "one size fits all" unique liposomal formulation with optimal adjuvant properties for any antigen has not been found, since these properties depend on the physicochemical characteristics of the vesicle (lipid composition, size, presence or absence of surface charge), the use of additional adjuvants or co-stimulatory molecules, and even the immunization schedule being used [2]. These problems have led to the suggestion that the potential of specific liposomal formulations as immunological adjuvants must be individually evaluated for each particular antigen.

One of the factors influencing the immunomodulatory properties of liposomes is their lipid composition. However, the reports on the specialized literature dealing with this subject are scarce, and their results are generally contradictory. This is therefore a very attractive and important research topic in the field of liposomal adjuvants.

The recombinant human epidermal growth factor (rhEGF) is a polypeptide having a high structural homology (70%) with its murine counterpart, being therefore a poor immunogen in rodents. It has previously been shown that the immunogenicity of rhEGF can be improved, both in animal models and humans, by its conjugation to tetanus toxoid or to P64k from *Neisseria meningitidis* [3-6], allowing the characterization of the immune response to this antigen and the development of a therapeutic vaccine for the treatment of tumors that depend on the EGF/R-EGF system. This vaccine contains rhEGF conjugated to P64k with Montanide ISA 51 or $Al(OH)_3$ (alum) as an adjuvant [7]. Since the liposomal vesicle may be a very effective vehicle for the presentation of poorly immunogenic or "self" antigens to the immune system, rhEGF thereby constitutes an ideal model antigen for their optimization because rhEGF is poorly immunogenic and due to the wide amount of information on the immune responses it induces under a variety of experimental conditions.

The steady increase of the incidence of allergic disorders in the world population, especially in developed countries, has called the attention of the scientific community. Dust mites (*Acari*) are the main causal agents of allergic diseases, and those of the *Dermatophagoides* genus are the main source of allergens in domestic dust. In particular, the *D. siboney* mite, endemic to the Caribbean, has been found in 85% of the dust samples collected from the houses of Cuban asthma patients. The main allergens of this mite (maDer s) are recognized by 80 to 90% of the sera from patients which are already allergic to this organism [8].

The first reports on the encapsulation of allergens in liposomal vesicles were published at the beginning of the 1990's. These studies were focused on the potentiation of allergen-specific immunotherapies, which is the most effective treatment for allergic affections. Allergies are characterized by the hyper-production of cytokines

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which are typical of a response mediated by CD4+ helper T-lymphocytes (Th2), and therefore those immunotherapeutic strategies favoring Th1 responses (using adjuvants that promote a Th1 response to the allergens) are most beneficial for treating these disorders [9]. Liposomes are safe and effective vehicles for this kind of therapy, since they enable the incorporation of immunomodulatory molecules such as microbial cytolysins together with the antigen, in order to steer the immune response towards a cell-mediated Th1 profile [10]. Our laboratory has previously studied the properties of Sticholysin II (StII), a cytolysin isolated from the *Stichodactyla helianthus* sea anemone that has a functional homology with bacterial pore-forming proteins. Therefore, the inclusion of StII into liposomes together with allergens (maDer s) might potentially modulate the immunomodulatory capacity of these vesicles.

With this in mind, the following objectives were proposed:

1. To evaluate the encapsulation and retention of rhEGF, conjugated or not to the P64k carrier protein, into liposomes with different phosphatidylcholines and obtained by several procedures, as well as to study the anti-rhEGF immune response induced in mice by these formulations compared to that produced by $Al(OH)_3$ as the conventional adjuvant.

2. To study the immunomodulatory capacity of liposomes containing the main allergens (maDer s) from *D. siboney*, and the effect of the inclusion of cholesterol or StII on their adjuvantic capacity.

Materials and methods

rhEGF and recombinant P64k from *Neisseria meningitidis* were obtained at the Center for Genetic Engineering and Biotechnology (CIGB) [11, 12]. The extracts of the *D. siboney* dust mite (internal reference DSB 7031) and the partially purified allergens (maDer s) from this extract were obtained at the Department of Allergens of the National Center of Biopreparations (BIOCEN). The Der s 1 concentration in both allergenic preparations was determined using a sandwich-type quantitative ELISA, with monoclonal anti-Der s 1 antibodies [13]. Sticholysin II (StII) was isolated and purified from the sea anemone *Stichodactyla helianthus* at the Center for Protein Studies (CEP) of the School of Biology, at the University of Havana [14].

Obtaining the liposomes

The dehydration-rehydration procedure [15] was used to prepare dimiristoyl-phosphatidylcholine (DMPC), dipalmitoyl-phosphatidylcholine (DPPC), distearoyl-phosphatidylcholine (DSPC), soybean phosphatidylcholine (sPC) or egg yolk phosphatidylcholine (ePC) and cholesterol (Cho) vesicles (DRVs, molar phospholipid:Cho ratio of 1:1). The proteins were added in the following proportions: phospho-lipid:rhEGF 32 µmol:300 µg (for liposomes containing rhEGF or rhEGF-P64k), phospho-lipid:P64k 32 µmol:30 µg, phospholipid:maDer s 16 µmol:36 µg (for DPPC liposomes) or 16 µmol:80 µg (for DPPC and DPPC:Cho liposomes). The vesicles of DPPC and DPPC:Cho containing StII were prepared with a phospholipid:maDer s:StII proportion of 16 µmol:80 µg:10 µg. To obtain large unilamellar vesicles (LUV), unpurified DPPC:Cho DRVs were extruded 5 times through 400 nm polycarbonate membranes at more than 45 °C.

MLVs were also prepared, using 16 µmoles of ePC and Cho and 0.025 µmoles of rhEGF. The vesicles, sonicated on an ultrasonic bath for 2 minute-intervals separated by 1-minute periods, received trehalose, sucrose, maltose or glucose in a sugar:phospholipid ratio of 0.5:1, and were later vacuum dehydrated for 24 hours. Their rehydration was performed with PBS.

DPPC:Cho FATMLV liposomes (molar ratio 1:1) were prepared by freeze-thawing [16], using 5 cycles of freezing in liquid nitrogen and thawing at 45 °C or more. The lipids were hydrated for 1 hour at T > 45° C with rhEGF, P64k, or rhEGF-P64k in PBS. The liposomes were separated from the unencapsulated material by ultracentrifugation, followed by two additional washes in PBS, and finally resuspension in the same buffer.

Protein labeling with ^{125}I

Proteins were labeled with ^{125}I by the chloramine T method [17]. The labeled proteins were rhEGF, P64k, maDer s and StII.

Preparation of rhEGF conjugates with P64k

rhEGF-P64k conjugates were prepared using glutaraldehyde a final concentration of 0.05% v/v) [3]. When using radiolabeled proteins, the conjugates were further purified from the reaction mixture by gel filtration chromatography on Sephadex G-50 medium grade, at 12 mL/h, in a 60 x 1 cm column equilibrated with PBS. The fractions from the first peak with the highest radioactive concentration were chosen for further studies. For experiments involving unlabeled proteins, such as immunizations, the conjugates were prepared in the absence of radionuclides and, at the end of the reaction, dialyzed against PBS for 24 hours.

Measuring the efficiency of protein encapsulation into liposomes

To determine the efficiency of protein encapsulation into DRV, FATMLV and LUV, the liposomes were prepared with radiolabeled proteins (rhEGF-^{125}I, P64k-^{125}I, rhEGF^{125}I-P64k, maDer s-^{125}I and StII-^{125}I) as tracers in a dose of 100 000 c.p.m., and the amount of unincorporated material was determined by measuring vesicle-associated radioactivity before and after their separation from unencapsulated proteins by ultracentrifugation.

Measuring of the retention capacity of the liposomes

The capacity of the liposomes to retain their encapsulated contents, was evaluated by preparing the vesicles with radiolabeled proteins and storing at 4 °C and, 24 hours post-encapsulation, or weekly for up to 1 month post-encapsulation; the radioactivity leakage into the solution was measured using the total radioactivity of an aliquot before and after removing the liposomes by ultracentrifugation.

Releasing rhEGF encapsulated into liposomes in the presence of plasma

A total of 80 mM of carboxyfluorescein (CF) were encapsulated in ePC:Cho (26 µmoles of total lipids, molar ratio 1:1) DRV with or without 0.02 µmoles

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of rhEGF. The liposomes were then mixed with fresh mouse plasma or PBS (as a control) using a liposome:plasma ratio of 1:5 (vol/vol). The CF released was measured in a Schimadzu RF-540 spectrofluorimeter (λ_{exc} = 490 nm, λ_{em} = 520 nm).

Measuring liposome size

Liposomal preparations were analyzed by photonic correlation spectroscopy, using a Malvern Autosizer 2C with a helium/neon 5 mW laser.

Analysis of liposomal phase transitions by high-sensitivity differential scanning calorimetry

DPPC and DSPC DRVs containing encapsulated rhEGF and control vesicles without rhEGF were prepared identically. One-hundred milligrams of each preparation were weighed and used to determine Tc and enthalpy change (ΔH) of the phase transition, using a high-sensitivity Micro-DSC III SETARAM differential scanning microcalorimeter.

Evaluation of the anti-rhEGF and anti-allergen immune responses induced with liposomes as adjuvants

Immunization experiments always used freshly obtained liposomal preparations. Balb/c and NMRI female mice from CENPALAB, aged 6 to 8 weeks and weighing from 18 to 20 g, were immunized intramuscularly (i.m.) or intraperitoneally (i.p.) using a 2-dose schedule: 0 and 28 days for rhEGF, or 0 and 14 days for maDer s. Blood samples were drawn either from the retroorbital cavity or by cardiac puncture.

Influence of the immunization schedule on the anti-rhEGF immune response

These experiments used Balb/c mice (6 animals/group). For liposome-immunized animals, DPPC:Cho DRV were prepared, encapsulating rhEGF or rhEGF-P64k. For $Al(OH)_3$-immunized animals, 2 mg of this adjuvant were used per dose, and the immunogen was prepared by mixing, under constant stirring for 30 minutes, 533 µL of $Al(OH)_3$ 30 mg/mL with 0.013 µmoles of rhEGF ($Al(OH)_3$/rhEGF) or a conjugate volume equivalent to 0.013 µmoles of rhEGF ($Al(OH)_3$/rhEGF-P64k). The titers of anti-rhEGF (IgG) antibodies in the plasma from the immunized animals were determined at days 14, 40 and 90, and the IgGs subclass at day 40.

Anti-rhEGF immune response induced by DRV liposomes of differing phospholipid composition

These experiments used NMRI mice (7 animals/group) immunized with 10 µg of rhEGF-P64k in DPPC:Cho, DSPC:Cho and sPC:Cho DRV. The control was $Al(OH)_3$/rhEGF-P64k, prepared by mixing 933 µL of $Al(OH)_3$ at 30 mg/mL with a conjugate volume equivalent to 0.023 µmoles of rhEGF. The IgG subclasses were determined 40 days after-immunization, and the capacity of the antibodies for inhibiting binding of rhEGF to its receptor (R-EGF) in human placental membranes was determined 90 days after-immunization.

Anti-maDer s immune response induced by DPPC liposomes with or without cholesterol and StII

Balb/c mice (6 animals/group) were immunized through the i.p. route with an amount of maDer s containing 5µg of Der s 1, adsorbed on $Al(OH)_3$ (600 µg/dose) or encapsulated into DPPC or DPPC:Cho liposomes. maDer s-specific total IgG, IgG1, IgG2a and IgE titers were determined at days 0, 14 and 28 post-immunization.

In another experiment, Balb/c mice (4 animals/group) were immunized similarly and then subjected to repeated allergenic challenges for 21 days after the first dose. This was achieved by placing the animals in a chamber (18 x 12 x 21 cm) and exposing them for 30 minutes, during 6 days, to aerosols of allergen extract solution from *D. siboney*, containing a Der s 1 concentration of 100 µg/mL. Twenty-four hours after the last challenge, the animals were bled to estimate eosinophile counts in peripheral blood, and their lungs were extracted for histopathological analysis.

The anti-maDer s humoral response in the presence of StII was studied by immunizing Balb/c mice (6 animals/group) with maDer s containing 5 µg of Der s 1, encapsulated together with StII into DPPC and DPPC:Cho liposomes. The StII dose received by the animals in each case was 1.4 and 1.3 µg, respectively. The animals were bled at day 28 after-immunization for determining the titers of allergen-specific total IgG, IgG1 and IgG2a.

Delayed-type hypersensitivity assay (DTH)

The delayed-type hypersensitivity assays (DTH) used 3 groups of NMRI mice (n = 10) immunized with 10 µg of rhEGF or rhEGF-P64k in DPPC:Cho DRVs or $Al(OH)_3$. The DTH was studied by challenging the animals in the pad of the left leg with 5 µg of rhEGF in PBS (vol. = 50 µL), 7 days after the second dose. Four animals from each group (control) were challenged with 5 µg of bovine serum albumin (BSA) in PBS. The inflammation was measured at 0 and 48 h post-challenge, using a 7150 UGO BASILE plethysmometer, and calculated as the difference between both readings. The histopathological analysis used a double hematoxylin-eosin staining.

In vitro cellular proliferation assay of mice lymphocytes

The *in vitro* cellular proliferation assay of mice lymphocytes used Balb/c mice immunized with 10 µg of rhEGF or rhEGF-P64k in $Al(OH)_3$ (2 mg/dose) or DPPC:Cho (1:1) DRV. The immunogens adjuvanted with $Al(OH)_3$ contained 0.015 µmol of rhEGF or rhEGF-P64k and 900 µL of $Al(OH)_3$ (30 mg/mL). Twelve days after the second immunization, the inguinal ganglia from 3 mice from each group were extracted and perfused, and the cells obtained were placed at a rate of 3 x 10^5 cells/well, in triplicate, to flat-bottomed 96-well Costar plates containing an RPMI 1640 culture medium supplemented with 10% fetal calf serum (FCT, Gibco). The cells were stimulated with 9, 18, 37 and 75 µg/mL of rhEGF, and cultured for 96 h at 37° C in a 5% CO_2 atmosphere. One µCi of thymidine -^{3}H (Amersham), was added 18 hours before the end of the culture. As a control for

basal cell growth, the proliferation induced by the culture medium alone in the absence of exogenous antigen and mitogen was also measured.

Antitumoral activity assay

Balb/c mice (12 animals/group) were immunized weekly, for 1 month, with 50 µg of rhEGF in $Al(OH)_3$ or 5 µg of rhEGF in DPPC:Cho DRVs using the intraperitoneal route (i.p). Control groups received only the corresponding adjuvant. The challenge was made with 2 x 10^5 cells of Ehrlich's ascitic tumor, 3 days after the last dose. The increase in life span was determined as the ratio between survival time in the immunized animals and in control animals.

Assay for inhibiting the EGF/R-EGF interaction by the antibodies from the sera of immunized mice

A total of 100 000 cpm of rhEGF-^{125}I were added to a suspension of human placental membranes containing 400 µg of proteins. After a one-hour incubation at room temperature with the sera from immunized or non-immunized control mice diluted 1:5, the reaction was stopped by including 1 mL of PBS, and centrifuged at 3000 g for 15 min. Binding inhibition produced by an excess (1 mg/mL) of unlabelled rhEGF was also measured as a positive control.

Estimation of eosinophile counts in peripheral blood and histopathological analysis of lungs

Twenty-four hours after the last treatment, 10 µL of blood were drawn from the retroorbital plexus of the animals challenged with *D. siboney* extract aerosols, and mixed with 200 µL of 1% eosin (Quimefa). The eosinophiles were counted on a Neubauer chamber. After euthanasia by cervical dislocation, the lungs were extracted to evaluate the presence of peribronchovascular inflammation by histopathology, using a double hematoxylin-eosin staining.

Passive cutaneous anaphylaxis assay

The levels of maDer s-specific IgE levels were evaluated using a passive cutaneous anaphylaxis (PCA) assay [18] on male Wistar rats (180-200 g body weight) from CENPALAB. Each serum sample was assessed on three rats, taken as replicates. Each rat received a subcutaneous inoculation on the dorsal region (previously shaved) of 100 µL of pooled sera (week 4) from immunized animals, diluted 1:5 in PBS. Forty-eight hours after-inoculation, the animals received an intravenous injection, through the penis vein, of 1 mL of allergenic extract from *D. siboney* (Der s concentration of 1.500 µg/mL) and 1% Evans Blue, diluted in PBS. The diameters of the stains in the skin of the dorsal region of each rat were averaged and analyzed as a criterion for the levels of specific IgE in their sera.

Assay for the titers of anti-rhEGF and anti-allergen antibodies by ELISA

The specific IgG levels for rhEGF or the allergens in the sera from immunized animals were evaluated using an indirect non-competition ELISA with the antigen directly immobilized on the solid phase. High-binding Costar 96-well plates were coated overnight at 4 °C with 50 µL of rhEGF at 10 µg/mL or 100 µL of *D. siboney* extract diluted to a Der s 1 concentration of 0.7 µg/mL, in 0.1 M Na_2CO_3/$NaHCO_3$ buffer, pH 9.6. Bound antibodies were detected using an anti-mouse IgG-peroxidase conjugate (Sigma), diluted 1:1 000, measuring absorbance at 492 nm on a Multiskan EX plate reader. The cut-off value for absorbance was of 0.2 or higher. The titers of IgG1, IgG2a and IgG2b were determined using a similar ELISA, but with an anti-subclass IgG-biotin conjugate (PharMingen) diluted 1:5 000, which was then detected with a streptavidin-peroxidase conjugate (PharMingen) at 1:2 000.

Determination of total IgE levels in the serum

To quantify total IgE levels in the serum, a sandwich ELISA was used following the recommendations of the manufacturer (PharMingen). The levels of IgE are given in micrograms per milliliter. The plates were coated with 100 ìL of a solution of the R35-72 monoclonal antibody against total mouse IgE, at a concentration of 2 mg/mL in coating buffer, overnight at 4 °C. The sera were added at a 1:20 dilution in the blocking solution, using 100 µL per well, in duplicates. Then, the wells received double serial dilutions of a reference IgE mouse monoclonal antibody, and the plates were incubated for 1 hour at 37 °C. After washing, 100 µL of a 2 µg/mL solution of biotinylated R35-95 (Anti-mouse IgE monoclonal antibody) were added.

Assay for protein concentration

The concentration of protein samples was determined by the Lowry method [19], as modified in our laboratory. The samples and standards, diluted in 1 N NaOH, received sodium deoxycholate at 5%. The reference curve was prepared by the dilution of a BSA stock solution at 1 mg/mL.

Statistical analysis

For the Statistical analysis of the data we used the Statistica software application package, version 6.0 (StatSoft, Inc., 2001). The assumptions of normality were tested with the Kolmogorov-Smirnov test, followed by the Bartlett or Levene tests to check for homogeneity of the variance. The presence of statistically significant differences between the means from different groups was determined using a single classification analysis of variance (ANOVA), with a model of fixed effects. Duncan's multiple-range test and the Student-Newman-Keuls tests were used for multiple comparisons. The means from two independent samples were compared with the t test; Mann-Whitney's U test was employed when the assumption on the homogeneity of variance was not met. The antitumoral assay used the Kaplan-Meier estimate for survival curves. Comparison of survival time between different treatments was made with the log-rank test as implemented in the statistical software package SPSS, version 10.05/1999 (Copyright SPSS Inc.). In all cases, a statistically significant result was assumed if $p < 0.05$.

Results and discussion

Results for rhEGF as the model antigen

The use of liposomal vesicles as the adjuvant requires optimizing the characteristics of this system for each

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particular antigen. In the case of rhEGF, the absence of literature on the use of liposomes for improving its immunogenicity implied that it was necessary to define the methodology for obtaing it and the lipid composition of determining liposomal preparations used. The best technological alternatives tested for the encapsulation of rhEGF, were given by the dehydration-rehydration method (DRV), as determined by measuring the amounts of encapsulated rhEGF and the capacity of the vesicles to minimize the leakage of the entrapped antigen during storage [20, 21].

The encapsulation experiments of rhEGF into DRVs, composed of different saturated or unsaturated phosphatidylcholines together with cholesterol in equimolar amounts, proved that the nature of the phospholipid is a basic factor in the encapsulation and retention capacities of these vesicles. The performance of DRVs for both parameters was consistently better when using dipalmitoyl phosphatidylcholine and cholesterol (DPPC:Cho) compared to liposomal preparations containing other phosphatidylcholines [20, 21].

The presence of a detectable leakage of rhEGF from DRVs during storage without concomitant changes in vesicle size suggested that this phenomenon might be caused by a direct interaction of the protein with the lipid bilayer, rather than by processes of liposomal fusion or aggregation. Indeed, the higher rate of release of carboxyfluorescein from vesicles containing encapsulated rhEGF, as well as the significant decrease observed in the ΔH of phase transition for DPPC and DSPC membranes containing rhEGF, showed that this polypeptide is interacting selectively with the phospholipids of the liposomal membranes, probably leading to the separation of polar groups in the interfacial zone of the bilayer and a decrease in the electrostatic interactions between the phosphorylcholine groups of adjacent phospholipid molecules [20, 21]. The release of rhEGF was decreased by the addition of sucrose, trehalose, maltose and glucose to the liposomal preparations [20, 22]. This protective effect has been attributed to a displacement by these sugars of the hydration water from the polar groups of the phospholipids, and the establishment of hydrogen bonds between their phosphate groups and the hydroxyls from the saccharides [23].

A second stage of this work studied the anti-rhEGF immune response in mice immunized with this molecule, either alone or as a conjugate to the P64k carrier protein, encapsulated into DRVs or adjuvanted with $Al(OH)_3$, which is a well-known conventional adjuvant. The immunization of Balb/c and NMRI mice with DPPC:Cho DRV containing rhEGF, whether alone or as a conjugate with P64k, induced high titers of anti-rhEGF IgG antibodies, with a predominance of the IgG1 subclass but also significant levels of IgG2a and IgG2b [24, 25].

The immune response obtained with rhEGF alone in DPPC:Cho DRVs was better than that of $Al(OH)_3$ as the adjuvant. Higher antibody levels (total IgG, as well as IgG1, IgG2a or IgG2b), better lymphocyte proliferation responses, and longer survival periods on challenging with Ehrlich's ascitic tumor cells were observed in animals receiving the liposome/rhEGF combinations compared to those receiving $Al(OH)_3$/rhEGF. Furthermore, the response induced by the encapsulation of rhEGF alone in DPPC:Cho DRV liposomes was similar to that obtained with rhEGF-P64k in liposomes or rhEGF-P64k adjuvanted in $Al(OH)_3$, whether considering total IgG levels, delayed-type hypersensitivity responses, or lymphoproliferation. These results suggest that the use of P64k as a carrier protein is not needed for the induction of an anti-rhEGF immune response when the antigen delivered is encapsulated into liposomal vesicles [24-26].

In the case of the rhEGF-P64k conjugate, the nature of the phosphatidylcholine used for the preparation of the encapsulating liposome affected the potentiation of the anti-rhEGF immune response, mainly in the quality of humoral immunity. The liposomes formed by saturated phosphatidylcholines (DPPC and DSPC) induced higher levels of IgG2a and IgG2b, and these antibodies exhibited higher binding inhibition levels of the rhEGF to its receptor compared with sPC (unsaturated) liposomes or $Al(OH)_3$, (Figure 1) [21, 25].

Results for maDer s as the model antigen

A first stage of this study was the encapsulation efficiency of maDer s and StII into DPPC and DPPC:Cho (molar ratio 1:1) DRVs. Two lipid:allergen ratios were tested, finding that the encapsulation yields for maDer s depend on this parameter. The presence of cholesterol in the vesicles also improved the encapsulation of both maDer s and StII.

The analysis of the retention of maDer s on the vesicles stored for a month in suspension at 4 °C

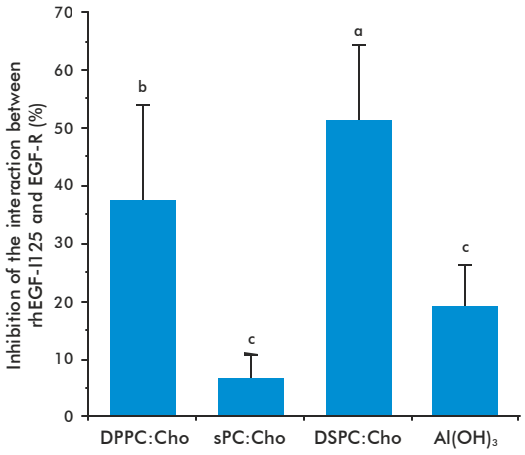

Figure 1. Inhibition of the interaction between EGF and its receptor by the sera of animals immunized with rhEGF-P64k encapsulated into DRV or adsorbed in $Al(OH)_3$. The vertical axis represents inhibition percentage of the rhEGF/R-EGF interaction due to the anti-rhEGF antibodies in the sera of immunized animals. Human placental membranes were used as the source of receptors. NMRI mice were immunized intramuscularly at days 0 and 28 with 10 μg of rhEGF conjugated to P64k. The assay used serum samples (n = 7) drawn 90 days post-immunization, diluted 1:5. The negative and positive controls were the sera of animals which had not been immunized and an excess of unlabelled rhEGF (assumed to produced a maximum degree of blocking or inhibition of the rhEGF-I^{125}/R-EGF interaction), respectively; these values were also used to calculate the inhibition percentages of the samples. Different letters represent statistically significant differences, according to Duncan's Multiple Range test ($p < 0.05$).

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revealed higher leakage rates when the vesicles also contained StII; this effect became more pronounced with the presence of cholesterol in the liposomal preparations. On the other hand, the retention of StII on DPPC:Cho liposomes was more efficient, suggesting that in this case there is a higher proportion of membrane-associated protein.

The study of the humoral immune response in Balb/c mice was centered in comparing the response induced by the allergens encapsulated into liposomes (LP/maDer s) to that induced by the allergens adjuvanted in alum (Al/maDer s) or just dissolved in PBS (PBS/maDer s). Both alum and liposomes induced a high allergen-specific total IgG response. However, the IgE levels in the sera of animals immunized with LP/maDer s were similar to those found in the control group only receiving PBS, and were much lower than those of the sera of animals immunized with the allergens adjuvanted in alum. The evaluation of the specific IgE response by a passive cutaneous anaphylaxis assay confirmed that the use of alum as an adjuvant favors the induction of an IgE response. A histopathological study of the lungs of animals previously subjected to an allergenic challenge showed reduced cellular infiltrates in the LP/maDer s group, similar to those of the PBS control and very different to those observed in the Al/maDer s, where the presence of inflammation was evident. These preliminary results suggest that the use of liposomes may be an effective tool in the immunotherapy of allergic diseases [27].

For a more through study of the allergen-specific immune response induced by these preparations, DPPC and DPPC:Cho liposomal vesicles were prepared, which also included the potential immunomodulatory protein StII. The presence or absence of cholesterol determined the characteristics of the maDer s-specific humoral immune response in terms of the amount of IgG1, IgG2a and IgE. The DPPC:Cho vesicles were better inducers of a non-allergic response profile (Figu-re 2). However, regardless the liposomal lipid composition, the immunization with the vesicles prevented the onset of eosinophilia in peripheral blood and peribronchovascular inflammation in the mice immunized and then challenged with aerosols of *D. siboney* extracts. The highest titers of maDer s-specific IgG2a (a subclass indicative of a Th1-type response) were obtained when using DPPC:Cho vesicles which also contained StII (Figure 2B). This is the first evidence of the immunomodulatory effect of this protein encapsulated into liposomes; showing, additionally, that this effect depends on the liposomal lipid composition.

The scientific novelty of this study can be listed as follows:

1. The DPPC:Cho DRVs have the highest efficiency for the encapsulation and retention capacity of rhEGF during storage.

2. rhEGF interacts with the phospholipids forming the liposomal vesicles.

3. It is possible to induce an immune response in mice with rhEGF encapsulated into liposomes, even in the absence of the P64k carrier protein.

4. The liposomes formed by saturated phosphatidylcholines are better inducers of a humoral response against soluble antigens such as rhEGF than the vesicles composed of unsaturated phosphatidylcholines or $Al(OH)_3$.

5. The liposomal vesicles potentiate the specific immune response against the main allergens of *D. siboney*.

6. It is possible to induce a non-allergenic, allergen-specific immune response by the inclusion of cholesterol in the DPPC vesicles.

7. The presence of the StII cytolysin encapsulated within liposomes together with the allergens potentiate the immunomodulatory properties of the DPPC:Cho liposomal vesicles.

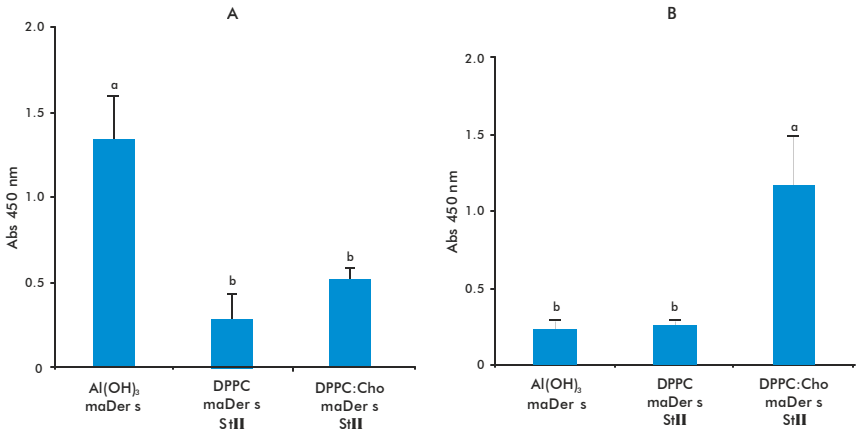

Figure 2. Levels of anti-allergen IgG1 (A) and IgG2a (B) (28 days) in Balb/c mice immunized with maDer s encapsulated, together with StII, into DPPC and DPPC:Cho DRV, or adsorbed with $Al(OH)_3$. The animals were immunized intraperitoneally at days 0 and 14 with an amount of maDer s containing 5 μg of Der s 1. The assay (ELISA) used serum samples diluted 1:100. Only the averaged values together with their standard deviations are shown (n = 6). Different letters above the bars indicate statistically significant differences between the experimental groups, according to the Student-Newman-Keuls test ($p < 0.05$).

According to scientific theory, the importance of this study lies in its contributions to the knowledge of the effect of factors such as the lipid composition of liposomes and the inclusion of other solutes (sugars, cytolysins) to the liposomal preparations on the efficiency of encapsulation and antigen retention in these systems, as well as in their immunomodulatory properties. From a practical perspective, this research validates the potential of liposomes as immunoenhancers for poorly immunogenic antigens -such as rhEGF- and the feasibility of their use in eliciting better allergen-specific immune responses. The adjuvant effect of liposomes with saturated phosphatidylcholines and cholesterol, prepared by dehydration-rehydration, was demonstrated by the encapsulation of a poorly immunogenic recombinant protein and the induction of an effective immune response without the need for conjugation to a protein carrier. These results may contribute to the improvement of the therapeutic rhEGF vaccine developed in our country by the Molecular Immunology Center by offering an attractive and promising alternative for presenting this antigen to the immune system without foreseeable limitations to its use in humans.

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versity in Brazil[4], and The School of Pharmacy, University of London, United Kingdom[5]: Dr. Anselmo Otero[1], Tech. Eric Felicó[1], Tech. Liem Canet[1], Dr. Gisela González[2], MSc. Irene Beau soleil[2], Dr. Edgardo Aníbal Disalvo[3], Dr. Dunia Rodríguez[4], Dr. Momtchilo Russo[4], and Dr. Gregory Gregoriadis[5]. We would also like to thank the University of Havana, the Ministry of Science, Technology and Environment (CITMA), the Royal Society and the Institute for Iberian-American Cooperation for funding the scholarships and projects that made this research possible.