

Liposome-entrapped *D. pteronyssinus* vaccination in mild asthma patients: effect of 1-year double-blind, placebo-controlled trial on inflammation, bronchial hyperresponsiveness and immediate and late bronchial responses to the allergen

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Summary

Background Allergen vaccination is effective in mite-allergic asthma. Liposomes are immunological adjuvants that can act as allergen carriers.

Objective To evaluate the immunological and functional effects of a liposome-entrapped *D. pteronyssinus* vaccine on mite monosensitive, mild asthma patients.

Methods A double-blind, placebo-controlled trial was conducted on 26 asthma patients who randomly received vaccination or placebo for 1 year. The levels of exposure to Der p 1 allergen were constant during the study. Allergen bronchial challenge was made at the beginning (T0) and after 1 year of treatment (T12). The day before and 24 h after the allergen provocation, patients were challenged with methacholine (Mth) (until FEV₁ fell by 40%) and blood and sputum samples were obtained. Dose–response curves to Mth were evaluated in terms of Mth-PD₂₀ (dose of Mth that induced 20% drop in FEV₁), slope (Mth-DRS) and level of plateau. Blood and sputum eosinophils and serum levels of eosinophil cationic protein (ECP) and intercellular adhesion molecule-1 (ICAM-1) were measured.

Results Groups were comparable at the start of the trial. At T12, previous to the allergen challenge, the active group showed higher values of both FEV₁ and Mth-PD₂₀ and lower values of Mth-DRS. The number of patients presenting a level of plateau increased in the active group (from two to four) and decreased in the placebo group (from two to one). At T12, before the allergen challenge, serum ECP levels increased in the placebo group and blood eosinophils showed a trend towards lower numbers in the active one. The immediate response and the changes in Mth-DRS values, sputum eosinophils and serum ECP levels following the allergen challenge were attenuated in the active group.

Conclusion Liposome-entrapped *D. Pteronyssinus* vaccination: (i) protects mild asthma patients from the worsening of asthma due to sustained mite exposure; and (ii) reduces the functional and inflammatory changes induced by allergen bronchial provocation.

Keywords allergen bronchial challenge, allergen vaccination, *Dermatophagoides pteronyssinus*, dose–response slope, eosinophilic inflammation, liposomes, maximal response plateau, mild asthma, randomized double-blind placebo-controlled trial

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Introduction

There is a wide range of experimental evidence supporting the view that inhaled indoor allergens are closely related to the development of airway inflammation, bronchial hyperresponsiveness (BHR) and asthma symptoms [1]. Allergen avoidance and anti-inflammatory drugs are the cornerstone of allergic asthma therapy [2], but these measures do not always adequately control the disease. In such instances, allergen vaccination (AV), that consists of administering gradually

increasing quantities of an allergen extract to an allergic patient [3], would be indicated [2].

The value of AV in improving symptoms and baseline lung function has been widely documented in perennial mite asthma [4]. The effect of mite AV on BHR is less clear as early studies reported no change in bronchial sensitivity [5], but others have documented a slow but constant improvement in BHR [6–8]. Reduction of eosinophilic inflammation in blood, skin, nasal mucosa or conjunctiva has been reported following AV [9], presumably due to its capacity to decrease Th2-cell activity (producing IL-4 and IL-5). It is not clear if such effect is secondary to a shift in the balance of T cell subsets in favour of a Th1-type response [9], or to the IL-10-dependent suppression of the specific peripheral T cell [10]. However, little is known about the effect of AV on bronchial airway inflammation. In this

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aspect, Kohno et al. [11] reported the results of an open trial in which mite AV reduced the numbers of eosinophils and the levels of eosinophil cationic protein (ECP) in sputum as well as the synthesis of IL-5 by T cells.

Natural [12] or experimental [13] exposure to the relevant allergen by asthma patients is frequently followed by an enhancement in eosinophilic inflammation and BHR, often accompanied by a late onset of asthma symptoms. It is well known that AV reduces the immediate allergen-induced bronchial response [6, 7] but data about the effect of mite vaccination on the late bronchial response are limited. In this regard, according to Kohno et al. [11], mite AV attenuates the allergen-induced increase in both eosinophilic inflammation and bronchial sensitivity to methacholine (Mth).

The above studies have evaluated the effect of bronchoconstricting agonists in terms of sensitivity, given by the provocative concentration or dose, needed to decrease FEV₁ by 20% (PC₂₀ or PD₂₀). However, BHR is a more complex functional disorder characterized by increased sensitivity but also by excessive airway narrowing (response) [14]. Following agonist inhalation, asthma patients exhibit ongoing degrees of airway narrowing without a maximal response plateau (MRP) being identified [15]. The lack of plateau or its detection at high degrees of bronchial obstruction has major significance in asthma, as it puts patients at risk for serious disease [16] and might indirectly resemble the inflammatory component of the bronchial wall [14]. To our knowledge, no report has studied the effect of mite AV on the bronchial response to bronchoconstricting agents.

Most studies evaluating the efficacy of AV have been performed with vaccines containing aluminium hydroxide gel as adjuvant. Liposomes are small vesicles of non-toxic, biodegradable and naturally occurring lipids, that act as potent immunological adjuvants with little allergenic potential [17], inducing a Th1-shift of the immune response [18]. Liposomes have a notable depot effect after subcutaneous injection [17] and increase the delivery of proteins to lymphatic vessels, lymph nodes and macrophages [19]. These properties suggest a role for liposomes as AV adjuvants.

We have used a vaccination extract in which controlled quantities of *Dermatophagoides pteronyssinus* (Dpt) major allergens (Der p 1 and Der p 2) were entrapped in liposomes. We designed a double-blind placebo-controlled (DBPC) trial in which Dpt-monosensitive mild asthma patients were randomized to receive either liposome-entrapped Dpt (LEDpt) or empty liposomes for 1 year. In a previous report we demonstrated that LEDpt treatment improved both clinical (reduction in symptoms, medication-consumption and days with symptoms) and immunological (reduction in IgE and increase in IgG₄) scores [20]. In the present study, we have evaluated the effect of LEDpt vaccination on:

- Lung function, bronchial sensitivity and response to Mth and blood and sputum eosinophilic inflammation. Dose-response curves to Mth (until FEV₁ fell by 40% or the highest Mth concentration (200 mg/mL) was inhaled) were analysed.
- Immediate asthmatic response to the allergen bronchial challenge.
- Allergen-induced changes in bronchial sensitivity and response to Mth and in blood and sputum eosinophilic inflammation.

To avoid results being attributed to other factors than AV, patients only received β -agonist drugs and mite exposure, confirmed by the analysis of Der p 1 levels, was constant throughout the trial.

Materials and methods

Patients and study protocol

Twenty-six non-smoking, Dpt-monosensitive, short-evolution (12–24 months) and mild asthma patients were randomized to receive injections of a LEDpt vaccine (active group) or empty liposomes (placebo group) for 1 year. In keeping with international guidelines [21], the diagnosis of asthma was based on a clinical history of recurrent attacks of wheezing, cough, breathlessness and chest tightness and only mild asthma patients were recruited. Sensitization to Dpt was verified by positive skin prick test with a standardized extract (ALK-Abelló, Madrid, Spain) and specific IgE (Pharmacia CAP System; Pharmacia Diagnostics, Upssala, Sweden) in the context of a compatible clinical history. Patients sensitized to other perennial or seasonal allergens were excluded. All subjects were informed and gave their written consent before starting the study. The ethics committee of our hospital approved the study protocol. The study design is shown in Fig. 1.

After selection, patients were instructed to perform simple mite avoidance measures at their dwellings, which included the minimization of mite allergen reservoirs and the reparation of plumbing and dampness problems. Mattress or pillow covers were not used. After a 4–6-week period (T0) and after the 12-month treatment period (T12), Der p 1 allergen levels were measured in dust samples from patients' beds.

The Dpt-bronchial provocation test (Dpt-BPT) was also made at both T0 and T12. On the day before and 32 h following the Dpt-BPT, subjects underwent clinical and physical evaluation and Mth challenge. Samples of peripheral blood and induced sputum were also obtained before and 24 h following the allergen challenge. Only short-acting β -agonist drugs used 'as needed' were allowed during the study. In the case of asthma exacerbation a short course of oral corticosteroids (up to a maximum of two courses) was advised. During the month previous to the study at both T0 and T12, patients had not presented airway infections and had not received corticosteroids.

Allergen extract

The same batch of an extract obtained from Dpt mite bodies was used throughout the study for treatment and *in vivo* tests. Total allergenic activity of the extract was measured by RAST inhibition against a reference extract biologically standardized [22]. The content of major allergens, Der p 1 and Der p 2, was quantified by a monoclonal antibodies-based enzyme immunoassay [23]. The extract, at 10 biological units/mL contained 4 μ g/mL of Der p 1 and 2 μ g/mL of Der p 2.

Liposomes composed of dipalmitoyl-phosphatidyl-choline, cholesterol and tocopheryl acid succinate (60:40:1 molar ratio) were prepared as described [24]. Dry lipid films were hydrated with either a solution of Dpt extract (active liposomes) or solvent alone (placebo liposomes). After vesicle formation, the resulting multilamellar liposomes were processed by

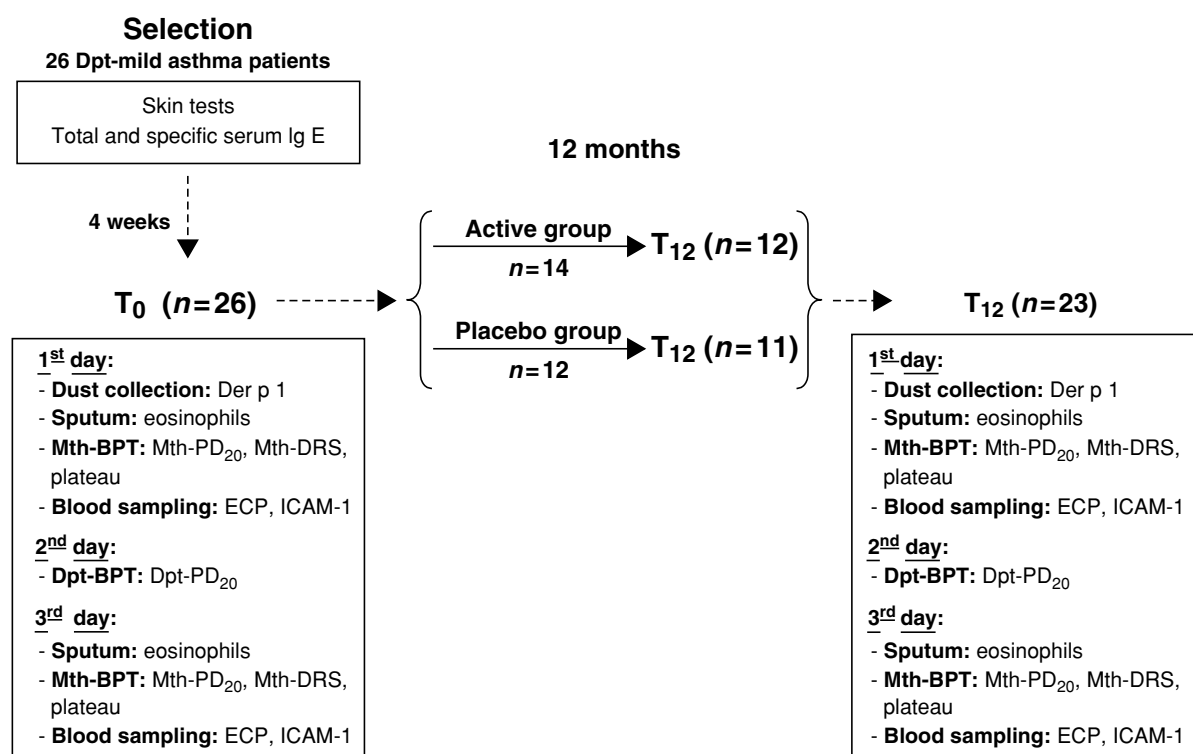


Fig. 1. Study protocol.

extrusion through 0.2 µg polycarbonate membranes (Millipore, Bedford, MA, USA) and non-encapsulated protein was removed by diafiltration. Liposome size and homogeneity were checked by photon correlation spectroscopy using a Coulter N4MD particle analyser. Lipids were measured using commercial kits (Boehringer, Mannheim, Germany) and major allergen content was determined by ELISA [23] after lysing the vesicles with sodium deoxycholate. Active liposomes were adjusted to the required antigen concentrations by dilution with the solvent. To make the placebo and active preparations equate, placebo liposomes were prepared at the same lipid concentrations as the active liposomes. Each vial was reconstituted before use and a constant volume of 0.5 mL was administered subcutaneously. After reconstitution, 90% of the allergen remained encapsulated in the vesicles (data not shown).

Treatment administration

Vaccination started between January and March 1997. During the up-dosing phase, the active group received eight weekly LEDpt extract injections, of 0.5 mL each, containing increasing doses of Der p 1 (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 µg of Der p 1/mL). When the highest or the maximum tolerated dose was reached, two maintenance doses were administered fortnightly and then the interval between doses was prolonged to 4 weeks. The placebo group followed the same schedule but received empty liposome injections prepared in identical vials (in appearance and lipid content) to the above.

Collection of dust samples and quantification of mite allergens

Dust collection was carried out as described [25]. A portable vacuum cleaner provided with a pre-filter (ALK, Copenhagen,

Denmark) [26] was used. The layer of bedding below the lower sheet, the layer above the upper sheet and inside the pillow case were vacuumed for 2 min/m². After vacuuming the filter box was removed and stored in a plastic bag at -20 °C until further analysis. The content of major mites' allergen (Der p 1) was determined in duplicate by a commercial ELISA based on monoclonal antibody (ALK). Standard curves for assays (1–200 ng allergen/mL) were routinely obtained from freeze-dried whole mite extracts tritiated against purified allergens. Allergen concentrations were estimated from three independent experiments. The mean intra-assay coefficient of variation was below 3%, while the interassay coefficient of variation from controls tested in all the assays was below 15%. The detection limit of the assays was 1 ng/mL. At T₀, median Der p 1-values were 4.2 and 4.4 µg/g of dust for the active and placebo groups, respectively, and remained stable throughout the study.

Bronchial provocation tests

Every lung function test was performed with the same spirometer (CPR; Medical Graphics Corp., St Paul, MN, USA). Reference values of Crapo et al. [27] were used. Baseline FEV₁ values ≥ 70% predicted normal were required to start tests. Patients did not inhale β-agonist drugs for the previous 8 h. The solution of Mth or allergen was then administered by a dosimeter (MEFAR s.r.l.; MEFAR, Borezzo, Italy) programmed to deliver five inhalations of 1 s each. The dosimeter was always attached to the same nebulizer calibrated to deliver 10 µL of solution in each second. Patients were carefully instructed to slowly inhale the solution from the residual volume to the total lung capacity and 10 µL of solution was administered in each breath. Before starting the BPTs, patients

inhaled diluent and a variability in FEV₁ values < 5% between baseline and post-diluent FEV₁ values was required.

Methacholine-BPT (Mth-BPT)

Mth-BPT was performed before and 32 h following the Dpt-BPT at both T0 and T12. Dilutions of Mth (Provacholine; Roche Laboratory, Nutley, NJ, USA) at 0.125, 0.25, 0.5, 1.0, 2.0, 5.0, 10.0, 25.0, 50.0, 100.0 and 200.0 mg/mL in PBS were made on the day. Methacholine at increasing concentrations was administered by the dosimeter, and the value of FEV₁ measured 3 min later. At each stage, the best FEV₁ value from three reproducible manoeuvres was recorded. The test continued until FEV₁ fell by 40% of the post-diluent value or the highest concentration was inhaled. Results were expressed in terms of the provocative cumulative dose of Mth, in μ mol, needed to decrease FEV₁ by 20% from the post-diluent value (Mth-PD₂₀), where 1 mol Mth = 195.4 g; the percentage of change in FEV₁ from baseline in response to cumulative dose of Mth inhaled (Mth dose-response slope, Mth-DRS) [28] and the level of plateau. A plateau was recognized if three or more of the highest doses of agonist fell within the 5% response range. The level of maximal response was obtained by averaging the data points on the plateau.

D. pteronyssinus-BPT (Dpt-BPT)

Dilutions of the Dpt extract at 0.04, 0.1, 0.2, 0.4, 1.0, 2.0 and 4.0 μ g Der p 1/mL in PBS were made on the day and inhaled at increasing concentrations provided by the dosimeter. FEV₁ values were recorded 10 min later. The test continued until FEV₁ fell by 20% from the post-diluent value or the highest concentration of allergen (4.0 μ g Der p 1/mL) was inhaled. During the next hour, spirometry was made every 10 min. At each stage, the best FEV₁ value from three reproducible manoeuvres was recorded. Results were expressed in terms of the provocative cumulative dose of allergen in ng of Der p 1 needed to decrease FEV₁ by 20% of the post-diluent values (Dpt-PD₂₀). The lowest FEV₁ value recorded during the 60 min following the allergen challenge was considered for analysis.

Sputum induction

Sputum was induced by hypertonic saline inhalation from an ultrasonic nebulizer (Ultraneb 99; De Vilbiss, Somerset, PA, USA) as described [29]. Saline at 5% was inhaled for three periods of 10 min. After each period, patients were asked to cough and expectorate into a sterile container, and saliva was, when possible, put away in a different receptacle. The test finished when a macroscopically adequate sputum sample was obtained or when the three inhalation periods were completed. The volume of the sample was measured and 3 mL of it were mixed with an equal volume of dithiotreitol (Sputasol; Unipath Ltd, Basingstoke, Hampshire, UK) at 1/100, and rocked at room temperature for 15 min. Then, the mixture was put through one 0.45- μ m filter (Millipore, Somerset, PA, USA) and centrifuged at 1500 g for 10 min. The pellet was suspended in saline at 0.9%, cytocentrifuged and stained with either Papanicolaou or Giemsa for differential cell counts. The sample was adequate when macrophages were visualized and squamous cell contamination was under 20% [29]. A single person blinded to the patients' clinical conditions made percentage counts of cells over a total count of 400 cells.

Serum inflammatory markers

Specific enzyme immunoassays were used to quantify the concentrations of ECP (UniCAP; Pharmacia Diagnostics) and intercellular adhesion molecule (ICAM-1) (T-Cell Diagnostics Inc., Cambridge, MA, USA). The limit of detection was 2.0 ng/mL for ECP and 0.3 ng/mL for ICAM-1.

Statistical analysis

The SPSS Windows 8.0 program (SPSS, Chicago, IL, USA) was utilized. Descriptive statistics were used to summarize the patients' clinical and demographic data and results expressed as median and interquartile range (IQR). Changes between the study periods were calculated as the absolute difference among the values. To obtain normal distribution of data, all the PD₂₀ values were log-transformed and results expressed in terms of geometric mean (gm). The changes in PD₂₀ values between the study periods were calculated as the ratio between the difference of logarithms and the logarithm of 2 and expressed in terms of doubling steps of concentration (DSC). Comparisons were analysed by the Mann-Whitney *U*-test and by the Wilcoxon's signed-rank test on paired data. A *P*-value lower than 0.05 was considered significant.

Results

Patients were randomly enrolled in the active (*n* = 14, 64% male) and placebo (*n* = 12, 67% male) groups (Fig. 1). The median age was 19.5 years (IQR: 18–24) and 20.5 years (IQR: 18–29), respectively. Twenty-three patients completed the trial: one subject from each group was withdrawn due to worsening of asthma and one patient in the active group left the study after 10 months due to personal reasons unrelated to AV (Fig. 1). Despite finishing the trial, two patients (one from each group) who presented bad tolerance to the allergen challenge at T0 did not consent to undergo the study at T12 [Fig. 1]. Vaccination with LEDpt was well tolerated and no severe reaction was presented. All the active patients reached the maximum expected dose of Der p 1 (3.2 μ g) and the cumulative dose received at T12 was $37.8 \pm 8.8 \mu$ g of Der p 1. Table 1

Comparative study before the allergen provocation test

Differences between groups at T0 (Table 1) The active and the placebo groups were comparable in terms of sex, age, baseline FEV₁ (median: 100% (IQR: 88–101) vs. 93.5% (84–107)) and sensitivity and response to Mth. A level of plateau was identified in three subjects from the active group and in four patients from the placebo one. Groups did not differ in blood eosinophils numbers, serum ECP levels or serum ICAM-1 levels but higher sputum eosinophilia was observed in the placebo group (*P* = 0.03).

Comparative study between groups at T12 (Table 1) The active group exhibited higher FEV₁ values (median: 106.5% (IQR: 98–110) vs. 96.5% (87.5–101), *P* = 0.034), higher Mth-PD₂₀ values (*P* = 0.034) and lower Mth-DRS values (*P* = 0.033) than the placebo group. A level of plateau was observed in four patients from the active group (two of them had identifiable plateau at T0) and in one patient from the placebo group who

Table 1. Functional and inflammatory indices obtained in the active and in the placebo groups at T0 and at T12. Results are expressed in terms of median and interquartile range (IQR), with the exception of Mth-PD₂₀ values (mean $\pm$ standard deviation)

	Active group				Placebo group			
	T0		T12		T0		T12	
	Pre-Dpt-BPT	Post-Dpt-BPT	Pre-Dpt-BPT	Post-Dpt-BPT	Pre-Dpt-BPT	Post-Dpt-BPT	Pre-Dpt-BPT	Post-Dpt-BPT
Mth-PD ₂₀ (μ mol)	0.97 $\pm$ 5.52	0.12 $\pm$ 4.15	3.41 $\pm$ 5.66	0.80 $\pm$ 9.56	1.04 $\pm$ 5.57	0.25 $\pm$ 4.99	0.35 $\pm$ 12.32	0.21 $\pm$ 9.32
Mth-DRS (%/ μ mol)	15.95 (1.5–44.4)	66.9 (30.0–100.3)	2.27 (1.23–6.99)	4.35 (1.87–7.66)	21.6 (3.65–72.9)	40.4 (3.9–102.1)	22.9 (3.1–164.4)	100.0 (3.6–213.6)
Blood eosinophils (c/mm ³)	476.6 (183.7–654.1)	643.2 (459.8–1023)	351.5 (164.4–424.9)	378.2 (206.4–503)	382.1 (297.4–566)	723.3 (484.8–1080)	374.0 (216–438)	627.2 (249.8–806)
Sputum eosinophils (%)	5 (2.2–13)	37 (21–57)	5 (2.5–24.5)	43.5 (25.7–57.7)	15 (7.7–45.7)	62 (45–72)	26 (8.2–40.5)	67 (58.5–81)
Serum ECP (ng/mL)	14.3 (5.8–24.9)	27.6 (20.3–34.7)	19.4 (11.1–44.6)	25.9 (14.2–46.8)	11.5 (8.7–17.8)	22.7 (13.8–45.7)	17.7 (13.1–30.7)	23.1 (21.3–35.7)
Serum ICAM-1 (ng/mL)	224.5 (195–261)	246.6 (205.5–265.3)	239.7 (167.9–277.1)	213.9 (184.8–286)	242 (221–279)	254 (211.4–286)	233 (220–272)	227.8 (202.8–253.5)

also presented a MRP at T0. One patient from the active group who had presented a level of plateau at T0 dropped the study and did not undergo bronchial challenges at T12. Three out of the four placebo patients presenting a level of plateau at T0 lost it. The small number of subjects presenting MRP did not allow comparative statistics.

Intragroup comparative study between the trial periods (T0–T12) (Table 1) Methacholine-PD₂₀ values increased in the active group and decreased in the placebo group, reaching a trend towards significance in the placebo group ($P=0.067$). The changes in Mth-PD₂₀ values (0.99 ± 1.88 DSC for the active group and -0.62 ± 2.46 DSC for the placebo one) statistically differed between groups (Fig. 2). Serum ICAM-1 levels increased ($P=0.037$) and blood eosinophils showed a trend towards lower numbers ($P=0.066$) in the active group, whereas serum ECP levels increased ($P=0.018$) in the placebo group.

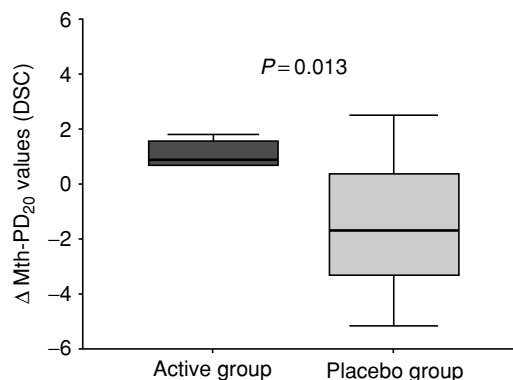
Bronchial response to D. pteronyssinus

Comparative study between groups at T0 No difference in the values of Dpt-PD₂₀ or in the cumulative doses of Der p 1 inhaled during the Dpt-BPT was detected [Fig. 3].

Comparative study between groups at T12 and intragroup differences between the trial periods At the end of the study, Dpt-PD₂₀ values increased 4 DSC in the active group and did not modify in the placebo group (Fig. 3a). At T12, the Der p 1 doses inhaled by the active group patients were nearly four times higher than those inhaled by the same subjects at T0 and nearly two times higher than those inhaled by the placebo group at the end of the study (Fig. 3b). In the placebo group, similar Der p 1 doses were inhaled throughout the trial (Fig. 3b).

Comparative study following Dpt-BPT: allergen-induced changes in functional and inflammatory indices

Differences between groups at T0 (Table 1) The Dpt-BPT reduced Mth-PD₂₀ in both groups ($P=0.002$ vs. $P=0.008$) and increased Mth-DRS in the active group (Fig. 4). The

**Fig. 2.** Comparison of the changes (Δ) in Mth-PD₂₀ values among groups (Mann–Whitney U -test).

allergen-induced changes in Mth-DRS values were higher in the active group (Fig. 5). The increments in eosinophil numbers in blood ($P<0.002$ for both) and sputum ($P=0.009$ vs. $P=0.015$) and in the levels of serum ECP ($P=0.005$ vs. $P=0.017$) observed following the Dpt-BPT did not differ between groups. After the allergen challenge, a level of plateau was detected only in one patient from the active group, who also exhibited it at the baseline.

Differences between groups at T12 [(Tables 1 and 2) The allergen challenge reduced the values of Mth-PD₂₀ in both groups ($P=0.028$ vs. $P=0.063$) and increased the values of Mth-DRS in the placebo group (Fig. 4). The allergen-induced increase in Mth-DRS values was higher in the placebo group (Fig. 5). After the allergen challenge, a level of plateau was identifiable in two patients from the active group (who also exhibited an MRP before the Dpt-BPT) and in no control subjects. In the active group, the numbers of eosinophils in sputum following the Dpt-BPT were lower than those exhibited by the placebo group ($P=0.010$). The active group exhibited lower allergen-induced increase in ECP levels than the placebo group (Fig. 6).

Fig. 3. Values of Dpt-PD₂₀ (a) and cumulative doses of allergen (Der p 1) inhaled during the Dpt-BPT (b) in the active and placebo groups at T0 and T12. Comparative study: Wilcoxon's signed-rank test on paired data (differences intragroup) and Mann-Whitney *U*-test (differences among groups).

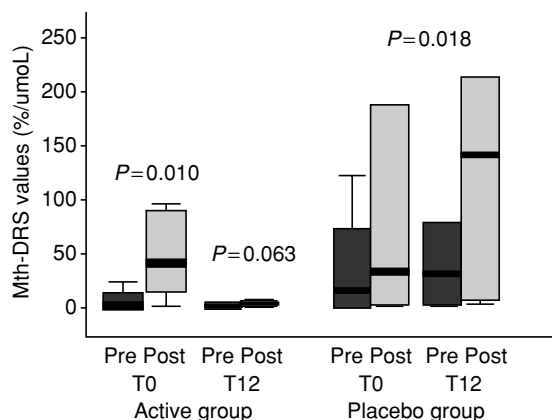
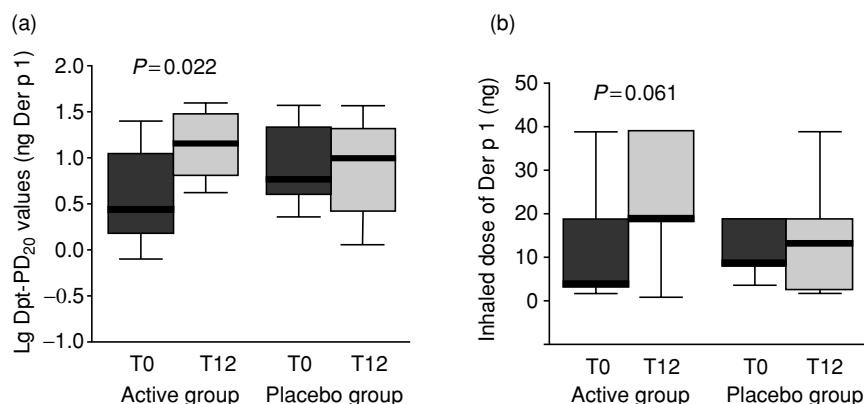


Fig. 4. Methacholine dose-response slope (Mth-DRS) values before and following the Dpt-BPT at T0 and T12 in the active and placebo groups. Comparative study: Wilcoxon's signed-rank test on paired data (differences intragroup) and Mann-Whitney *U*-test (differences among groups).

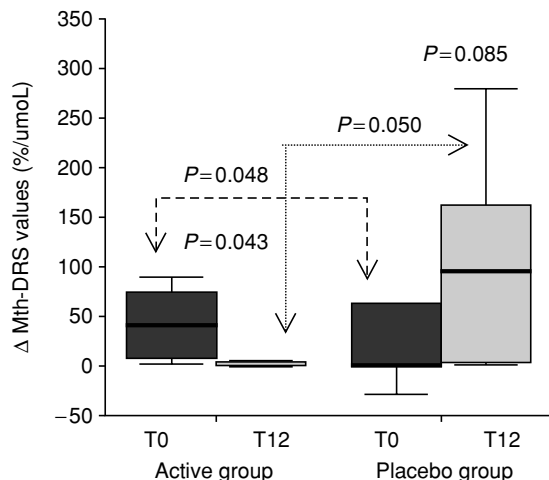


Fig. 5. Degree of changes (Δ) induced by Dpt-BPT in Mth-DRS values at T0 and T12. Comparative study: Wilcoxon's signed-rank test on paired data (differences intragroup) and Mann-Whitney *U*-test (differences among groups). The active group showed higher allergen-induced increases in Mth-DRS values than the placebo group at T0. When compared at T12, these changes were higher in the placebo group. When the study periods were compared, the allergen-induced increases in Mth-DRS values lowered in the active group and showed a trend towards higher values in the placebo group.

Intragroup comparative study between the trial periods (T0-T12) (Table 2) The allergen-induced changes in Mth-DRS values lowered in the active group and exhibited a trend towards an enhancement in the placebo group (Fig. 5). In the active group, the allergen-induced increase in serum ECP levels lowered at the end of the trial (Fig. 6).

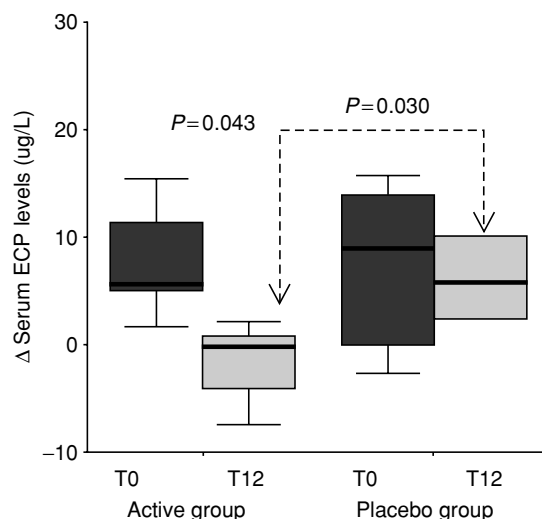
Discussion

Liposomes are non-toxic and biodegradable vehicles with the ability to both sustain release of multiple therapeutic agents, including allergens, and to bias the immune response towards a Th1-type [17-19]. These features raise the possibility of using liposomes as AV adjuvants. In this DBPC trial we have evaluated the effect of a 1-year treatment with a LEDpt vaccine on several asthma-related variables. To prevent results being attributed to other factors than AV, only short-acting β -agonist drugs were allowed and mite exposure, as verified by Der p 1 quantification, was stable during the trial. In the case of asthma exacerbation, short courses of oral corticosteroids were advised, but they always were at least 1 month apart from T12. Given the features of the trial and to avoid, as far as possible, the influence of irreversible airway structural changes secondary to the remodelling process, only mild and short-evolution (12-24 months) asthma patients were recruited. In spite of this, two subjects dropped the study due to asthma exacerbation.

Bronchial hyper-responsiveness is a complex functional disorder defined by dose-response curves. The shift of this curve to the left (PD₂₀) implies enhanced sensitivity, whereas its shape (response) denotes the extent to which the airways are capable of narrowing before reaching a plateau at which higher doses of stimulus do not further decrease FEV₁ [15]. The lack of identification of such a plateau is presumably the major pathophysiological abnormality in asthma as it puts subjects at risk for serious disease [16]. In most asthma patients a level of plateau is not identifiable even after inducing high degrees of airway narrowing [15] and in these cases the slope of the dose-response curve constitutes an approach to the evaluation of the bronchial response [16]. Mechanisms underlying bronchial sensitivity and response to bronchoconstricting agents are thought to be different; while sensitivity mainly depends on the integrity of the epithelium, the response is secondary to both the reduction in the forces that limit airway narrowing and the increase in

Table 2. Degree of changes (Δ) induced by the Dpt-BPT in functional and inflammatory indices in both groups at T0 and T12. Results are expressed as median and interquartile range (IQR), with the exception of the Mth-DRS values (geometric mean $\pm$ standard deviation)

Active group	Placebo group			
	T0	T12	T0	T12
Δ Mth-PD20 (DSC)	-3.65 ± 2.75	-3.03 ± 2.14	-2.12 ± 2.16	-1.31 ± 1.23
Δ Mth-DRS (%/ μ mol)	54.8 (5.7–77.8)	3.25 (0.08–5.08)	1.03 (–2.3–43.4)	56.6 (2.2–163.3)
Δ blood eosinophils (cel/mm ³)	244.65 (71.8–422.3)	83.15 (22.9–309.1)	298.5 (212.1–555.4)	83.2 (–146.7–278.9)
Δ sputum eosinophils (%)	15 (11–55)	28 (18–63)	26 (24–53)	35 (29–74)
Δ serum ECP (ng/mL)	11.44 (4.56–20.78)	–0.2 (–6.05–1.46)	13.95 (2.95–37.65)	7.65 (3.0–11.1)

**Fig. 6.** Degree of changes (Δ) induced by Dpt-BPT in serum ECP levels at T0 and T12. Comparative study: Wilcoxon's signed-rank test on paired data (differences intragroup) and Mann–Whitney *U*-test (differences among groups). At T12 the active group exhibited lower allergen-induced increases in serum ECP levels than at T0. These changes were also lower than those observed in the placebo group at T12.

airway wall thickness [16, 30]. Reductions in bronchial sensitivity following the treatment with high quality mite extracts have been described [6–8], but little is known about the effect of AV on bronchial response. When we compared the study periods, bronchial sensitivity and response to Mth decreased in the LEDpt-treated group and increased in the control group. However, these changes only reached statistical significance when groups were confronted at T12, which is likely to be secondary to the small size of the samples. Despite studying mild asthma patients and in keeping with previous reports [15], a level of plateau was not identifiable in most cases. Interestingly, no patient from the LEDpt group lost the MRP during the study and a level of plateau was also identified in two additional LEDpt-treated subjects. In contrast, two out of the three control subjects who had an identifiable MRP at T0, lost it at the end of the trial, suggesting that LEDpt-AV might play a protective role in the BHR increase due to mite continuous exposure [12, 31].

There is wide evidence to suggest that a misbalance between the T cell subsets in favour of a Th2-type T lymphocyte response is crucial in the development of inflammation in asthma [9]. Recent data suggest that AV works by shifting such anomalous immune responses towards a Th0 or a Th1 response, which results in the reduction of the synthesis of Th2 cytokines, IL-5 [11], IL-4 and IL-13 [32], responsible for eosinophilic inflammation and IgE synthesis [10]. In keeping with these findings, a reduction in eosinophils and in ECP levels in peripheral blood and target organs has been described following AV [9, 11]. Our study was performed on short-evolution and mild asthma patients who received no anti-inflammatory treatment. Thus, it is possible that, as described in seasonal respiratory allergy [33], LEDpt vaccination worked by preventing the enhancement in eosinophilic inflammation secondary to continuous allergen exposure [12]. In keeping with this, a trend towards lower blood eosinophilia in the LEDpt-treated group and statistically higher serum ECP levels in the placebo group were observed.

In this DBPC trial we could confirm previous studies [6, 11], carried out with aluminium hydroxide-containing vaccines, according to which AV reduces the immediate response of the target organ (bronchial mucosa) to the allergen. Besides the immediate asthmatic response, the inhalation of the relevant allergen by asthma patients enhances both BHR and eosinophilic inflammation [13, 29] and in some instances is accompanied by a late onset of asthma symptoms. The effect of AV on this late bronchial response has been scarcely evaluated. The allergen challenge increased bronchial sensitivity and bronchial response to Mth in both groups at T0 and at T12. At the end of the trial and despite the higher doses of Der p 1 inhaled during the allergen challenge, the LEDpt-treated group exhibited lower increases in both Mth-DRS values and serum ECP levels than either those observed at T0 or those presented by the control group at T12. In keeping with the larger allergen-induced increase of serum ECP levels, the control group showed higher numbers of sputum eosinophils at T12 following allergen inhalation. Possibly, the acute increase in bronchial eosinophilia might resemble an enhancement in the inflammatory infiltrate of the bronchial wall and consequently in its thickness that would lead to a higher degree of airway narrowing for the same muscle shortening. The intragroup and intergroup differences described above for Mth-DRS values were not detected in Mth-PD₂₀ values, supporting the assumption of different

factors underlying both sensitivity and response to bronchoconstricting agonists [16].

Intercellular adhesion molecule-1 is essential for leucocyte recruitment into the inflammatory focus but the meaning of its detection in biological fluids is not clear. The increase in serum ICAM-1 levels observed in the treated group at the end of the study might suggest an anti-inflammatory role for this molecule by blocking the endothelial receptor for ICAM-1 [34]. In our study, ICAM-1 values were not modified by the Dpt-BPT at either T0 or T12, which is in contrast to other authors [35]. Possible reasons for such lack of response might be the small size of the sample or the time of analysis (24 h following the allergen challenge), at which ICAM-1 values could have returned normal.

Finkelman et al. have warned about the possible risks of the pharmacological shift in the balance of Th-cell subsets by either decreasing the Th2 (worm infections) or increasing the Th1 (Th1-cytokine mediated inflammatory disorders) responses [36]. Although it seems very unlikely that specific allergen vaccination, widely used for a long time, might originate these problems, further studies are required to determine the long-term lack of adverse side-effects of liposomes when used as adjuvants for allergen-specific vaccination.

Taken together our data suggest that the treatment with liposome-entrapped *D. pteronyssinus* vaccination in highly selected (young, monosensitive, mild and short evolution) asthma patients:

- Prevents mild asthma patients from the deleterious effect of continuous mite allergen exposure on the airways: increase of both eosinophilic inflammation and BHR (sensitivity and response to methacholine).
- Reduces the bronchial immediate response to the allergen and increases the dose of allergen needed to inhale in order to elicit an asthmatic response.
- Attenuates the allergen-induced increases in blood and sputum eosinophilic inflammation and in bronchial response to methacholine.

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