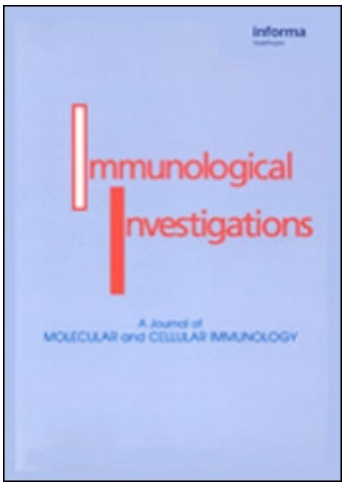

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Asymmetric IgG Antibodies Induced by Different Immunotherapies in a Murine Model of Allergy

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Specific immunotherapy (SIT) is the only potentially curative treatment for those allergic processes mediated by IgE. We compared the effects of different SITs in mice sensitised with ovalbumin (OVA) Al $(OH)_3$: 1) OVA entrapped in particles of poly (D,L-lactic-co-glycolic acid) (PLGA-OVA), 2) Soluble OVA (OVA-sol) and 3) Polymerised OVA (OVA-pol). Serum levels of specific IgE, IgG1, IgG2a and asymmetric IgG, the cutaneous anaphylaxis test (PCA), and the IL-10, IFNγ and IL-4 levels in culture supernatants of splenocytes challenged with OVA were assessed. Mice treated with PLGA-OVA had higher levels of asymmetric antibodies than non-desensitised mice; a low IgG1 and high IgG2a level was observed together with inhibitory effect in the PCA reaction that reversed in the absence of asymmetric IgG. IL-10 and IFNγ levels were higher in supernatants from mice treated with PLGA-OVA and OVA-sol than those obtained from non-desensitised controls. Our results suggest that among the different SITs evaluated, PLGA-OVA is the one that best showed an increase in the asymmetric IgG molecules and an effective deviation of the immune response. Furthermore, the increase in the proportion of asymmetric antibodies would be of importance when designing new vaccination strategies for allergy.

Keywords Asymmetric IgG, immunotherapy, allergy, PLGA.

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INTRODUCTION

Taking into account that the prevalence of allergic diseases has increased over the last years, the development of an effective therapy has become an important goal. Most of the current treatments are aimed at the reduction of the clinical symptoms of allergy. Specific immunotherapy (SIT) is, to date, the only effective and potentially curative treatment for those allergic processes mediated by IgE. Such SITs consist in the gradual administration, to an allergic individual, of increasing quantities of an allergenic vaccine. This sort of vaccine leads to the reduction of the symptoms caused by a subsequent exposure to the allergen (Bousquet, 1998; Malling, 1998; Durham, 1999; Scholl, 2005).

It has long been known that Th2 lymphocytes and mast cells synthesise IL-4 and IL-13, which favour the switch to IgE (Scholl, 2005; Filchelman, 2005). Many studies have demonstrated that SIT induces a deviation of the immune response towards a Th1 phenotype (Bousquet, 1998; Durham, 1999; Ciprandi, 2004) as well as an increase in the levels of anti-allergen specific IgG (Bousquet, 1998). Particularly in humans, the increase of IgG4 levels is one of the most important features: this immunoglobulin has an IgE-blocking activity (Wachholz, 2004; Ejrnaes, 2004).

It is also known that the chemical composition of antigen is crucial for the capacity of a vaccine to determine the type of immune response developed, regarding both the isotype of immunoglobulin synthesised and the cytokine profile generated (Storni, 2005).

Over the last years, a great deal of vaccine release systems have been developed employing synthetic formulations, such as those made of polymers of lactic and glycolic acids which are biocompatible and biodegradable (Coobes, 2004; Postlethwait, 2005). These systems release their antigenic contents in a controlled fashion, thus mimicking the repeated injections employed in the conventional schemes of vaccination (Johansen, 2000) and having the additional advantage of lack of toxicity (Wise, 1987). At present, these polymers are being used as allergen delivery systems (Batanero, 2002, 2003; Guerin, 2002; Igartua, 2001; Roth-Walter, 2005; Scholl, 2004; Sharif, 1994).

Furthermore several studies have demonstrated that 15–20% of the IgG molecules present in the serum of various species are asymmetric (Margni, 1998, 1972, 1973). Asymmetric IgG molecules are characterized by the presence of a mannose-rich oligosaccharide inserted in one of the Fd fragments of the heavy chain of this immunoglobulin. This prosthetic group causes steric hindrance, reducing 100 times the affinity of one of the binding sites of the antibody for its antigen. Experimental data demonstrate that the difference in the affinity for the antigen displayed by both binding sites of the asymmetric IgG is not due to an allosteric negative effect caused by the interaction of one of the binding sites with the ligand, since two populations of Fab fragments with different affinity can be isolated from the F(ab')2 fragment of the IgG

molecule (Leoni, 1986). As a consequence, these antibodies behave as monovalent and antigen-blocking, preventing the immune effectors mechanisms of the immune response from activation (Margni, 1998). In addition, unlike what is observed with soluble antigens, when a particulate antigen is inoculated, an increase in the proportion of asymmetric IgG is observed (Apicella, 2006; Canellada, 2002; Gentile, 2004; Labeta, 1986; Margni, 1983, 1986, 1998).

Many authors hypothesised that SIT mechanisms involve the induction of IL-10 and TGFβ producing CD4+ CD25+ regulatory T-cells inducing an anergic or tolerant state in peripheral T-cells (Ejrnaes, 2004).

Taking into account all these observations, in the present study we compared the effect of different specific immunotherapy in ovalbumin-sensitised mouse models. In order to evaluate efficacy, we measured established serum markers (IgE, IgG1, IgG2a) in combination with new parameters (asymmetric IgG) and we additionally performed ovalbumin stimulation assays on splenocytes from treated mice in vitro.

MATERIALS AND METHODS

Preparation of Microspheres

The microspheres of D,L-PLGA (poly-[D,L-lactic-coglycolic acid]) 50:50 (MW 50,000–75,000, Sigma, St. Louis, Mo) containing ovalbumin (OVA, Fluka, Switzerland) were prepared using a water-in-oil-in-water (A1/O/A2) emulsion based solvent evaporation technique (Chen; 2002).These hydrophilic particles of PLGA (50:50) can induce higher antibody levels than hydrophobic particles do (Johansen, 2000).

Determination of the Active Principle Content

Microspheres were treated with a solution containing 0.1N NaOH and 5% w/v sodium lauryl sulphate during 16 h at room temperature. Samples were then centrifuged (1000 *g*) and the protein content was measured in the supernatant employing the bicinchoninic acid method (BCA, Pierce, Illinois, USA). The OVA concentration in the samples was derived from a standard curve (Chen, 2002). Samples were analysed in triplicate.

Morphology and Size Distribution of the Particles

The morphological characteristics of the microspheres were evaluated by scanning electron microscopy (JEOL, JSM-35C, Japan) in a microscope equipped with a digital image acquisition system (SemAfore). Samples were adhered with a double phase adhesive tape to metallic slides and covered with gold by spluttering and employing a VE-300 VEECO evaporator operating under an argon atmosphere. The determination of the particle size was done

employing a light microscope (Arcano XSZ-107E, Argentina) measuring the diameters of 300 particles (Vila Jato, 1997). The distribution of the particle size was adjusted according to the frequency percentage.

Characteristics of the Microspheres

The microspheres prepared had an average size of 10.8 μm. Small particles (< 10 μm) are readily taken up by antigen presenting cells, thus stimulating the primary immune response, whereas large particles (> 10 μm) release the antigen to extracellular fluid, thus facilitating the generation of secondary antibody responses (Johansen, 2000). The protein content was 37.1 μg of OVA/mg microspheres.

Preparation of Polymerised Ovalbumin

Polymerised ovalbumin (OVA-pol) was prepared as described by Hayglass, 1991. Briefly, glutaraldehyde (6% in saline) was added dropwise to OVA at 25 mg/ml in 0.1 M acetate buffer, pH 5.3, 0.5 pH units above its isoelectric point to obtain a final molar ratio of 200:1. After dialysis and gel filtration (Bio-Gel A 50m, Bio-Rad Laboratories, Mississauga, Ontario, Canada) the polymerised protein (OVA-pol) was recovered as a single, sharp, symmetric peak of average Mr of 3.5×10^7 (Hayglass, 1991).

Sensitisation

Six-week-old Balb/c mice were sensitised with 3 intraperitoneal injections on days 0, 14 and 28, with 10 μg of OVA adsorbed in Al $(OH)_3$ in a final volume of 400 μl/animal. Non-sensitised control animals received Al $(OH)_3$ under the same conditions. Serum samples were taken on day 0 and on day 50. At the end of the sensitisation protocol (day 50) the allergy status was evaluated serologically by the determination of individual serum specific IgE, IgG1 and by the passive cutaneous anaphylaxis test in mice taken at random. These practices were performed following protocols for experimental animal use approved by the NIH.

Desensitisation Scheme

Sensitised mice on day 50 were divided into groups to be subjected to different desensitisation schemes: 1) PLGA-OVA (50 μg of OVA entrapped in PLGA in 100 μl of saline), 1 dose sc on day 50; 2) OVA-sol (50 μg in 100 μl of saline), 3 doses sc on days 50, 57 and 64; and 3) OVA-pol (50 μg in 100 μl of saline), 3 doses sc on days 50, 57 and 64, as model of particle antigen inducer of high IgG asymmetric antibodies. Control animals were included: sensitised mice treated with PLGA, 1 dose sc on day 50, and sensitised mice treated with saline solution on days 50, 57 and 64, respectively. Animals were killed on day 85.

Serum samples were frozen at –20°C for specific IgE, IgG asymmetric antibodies, IgG1, IgG2a and PCA determination. Spleens were removed and cultured.

Determination of Specific anti-OVA Antibodies (IgE, IgG1, IgG2a)

Levels of specific IgE, IgG1 and IgG2a were determined in serum samples obtained on days 50 and 85 by indirect ELISA. Briefly, Maxisorp polystyrene plates (Nunc Immunoplates, Rochester, NY, USA) were coated with OVA at 20 μg/ml in carbonate buffer pH 9.6 for 2 h at 37°C. Plates were then washed with PBS. As blocking reagent, a 10% non-fat dried milk solution in PBS was employed. After washing, suitable serum dilutions in PBS plus 0.05% Tween-20 (PBST) were added. Samples were incubated overnight at 4ºC. After washing, the following antisera were added: a goat anti- mouse-IgE (Bethyl Laboratories Inc., Montgomery), a goat anti-mouse IgG1 (Bethyl), a rabbit anti- mouse IgG2a (DAKO, Denmark) and a peroxidase conjugated goat anti-mouse immunoglobulins (DAKO, Denmark). After washing and for detection of IgE and IgG1, a biotinylated anti-goat antibody was added, followed by an avidin-peroxidase solution (Vector Laboratories, Inc, Burlingame, USA). For the detection of IgG2a, a peroxidase conjugated anti-rabbit serum was added. To measure levels of specific IgG, a peroxidase conjugate anti-mice IgG was employed. The reaction was developed by addition of a solution of o-phenilendiamine in citrate buffer pH 5.0 plus 0.004% H2O2. Optical densities were read at 490 nm. In all cases, the samples that were compared were tested at the same time and under the same conditions.

Determination of Specific anti-OVA Asymmetric IgG Antibodies by Concanavalin a Affinity Chromatography

Levels of specific asymmetric IgG were determined on days 50 and 85. Considering that α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues are present in the asymmetric IgG molecule, the percentage of this type of IgG was determined in sera by Concanavalin A (Con A)-Sepharose affinity chromatography (Amersham, Buckinghamshire, UK). Briefly, Con-A-coated Sepharose beads were washed with Con A buffer (0.025M Tris-ClH, 0.2M NaCl, 0.003M CaCl2, 0.003M MgCl2 and 0.003M MnCl2, pH: 7.2). Serum samples were mixed separately with equal amounts of Con A-Sepharose to determine symmetric IgG (IgG unbound to ConA) or with Buffer Con A to determine total IgG and were kept for 2 h at 4°C; the mixtures were then centrifuged and the specific IgG was determined in supernatants by ELISA. Briefly, Maxisorp polystyrene plates (Nunc Immunoplates, Rochester, NY, USA) were coated with OVA at 20 μg/ml in carbonate buffer pH 9.6 for 2 h at 37°C. Plates were then washed with PBS. As blocking reagent, a 10% non-fat dried milk solution in PBS was employed. After washing, suitable sample dilutions were added. For the detection of specific IgG, a

peroxidase conjugated anti-mouse IgG was added. The reaction was developed by adding a solution of o-phenilendiamine in citrate buffer pH 5.0 plus 0.004% H_2O_2. Optical densities were read at 490 nm. The percentage of asymmetric IgG was calculated considering the following formula: % bound IgG = 100–[(unbound IgG/total IgG)] × 100.

Cutaneous Anaphylaxis Test

Sera containing anti-OVA anaphylactic antibodies (IgE and IgG1) obtained from sensitised mice (day 50), and after different treatments (day 85), with or without Concanavalin A-sepharose adsorption, diluted 1/100 in saline were inoculated intradermically in the back of normal Balb/c mice (sera from 3 mice for each group were tested). After 2 h, 100 µl of a solution containing 20 µg OVA in Evans blue (5 mg/ml) were injected intravenously through the vein of the tail. A blue-coloured spot present in the antigen inoculation site >3 mm was considered a positive response (Dearman, 2003, 2005). The diameter of the blue spot was measured on the underside of the skin and the results were expressed as: Mild + = 3–4 mm and low blue spot; Moderate ++ = 5–10 mm and moderate blue intensity; High +++ > 10 mm and intense blue spot.

Splenocytes Culture

Determination of IL-10, IL-4 and IFN-γ. On day 85, spleens obtained from mice were removed under sterile conditions. Cell suspensions were prepared in RPMI 1640 supplemented with 10% of heat-inactivated foetal calf serum (Gibco, USA), antibiotics (penicillin 50 IU/ml and streptomycin 50 µg/ml), 2 g/l sodium bicarbonate, 2 mM glutamine and 1 mM pyruvate. Aliquots of 100 µl of the suspension containing 2.10^5 cells/ml were placed in 96 well U-shaped bottom microtitre plates and incubated with 100 µl of a solution of 1 mg/ml OVA, 100 µl/well of a solution of 10 µg/ml of Concanavalin A (positive control) or RPMI 1640 alone (baseline). After incubation for 96 h at 37°C with 5% CO2 in air, supernatants were collected and kept at –40°C for determination of cytokine levels by ELISA (R&D System, Minneapolis, USA) following the manufacturer's directions. Plates were read on an ELISA reader (Meterterch (Σ960) at a dual wavelength of 450–600 nm. Concentrations of each cytokine were derived from standard curves.

Statistical Analysis

All analyses were performed using one-way analysis of variance followed by Newman-Keuls multi-comparison test. In the case of asymmetric antibodies, IgG1 and IFNγ analyses were performed considering a logarithmic transformation of the data to satisfy ANOVA assumptions. The data were expressed as mean ± SE.

RESULTS

Immunologic Assessment of OVA-sensitised Mice

The sensitisation protocol induced high levels of OVA-specific IgE (OD 490 nm, day 50: 0.378 ± 0.020 vs day 0: 0.023 ± 0.040) and high specific IgG1 antibodies related to Th2 immune response (OD 490 nm, day 50: 1.506 ± 0.044 vs day 0: 0.011 ± 0.003). Sera from mice sensitised with OVA gave a high PCA reactivity score (day 50 :+++ > 10 mm and intense blue spot).

Immunologic Assessment of OVA-desensitised Mice

Levels of Specific Asymmetric IgG Serum Levels. We assessed the percentage of asymmetric antibodies induced by particulate and soluble desensitisation treatments. Mice treated with particulate antigens had higher levels of specific asymmetric IgG (PLGA-OVA: 39.2 ± 0.9%, and OVA-pol: 38.4 ± 4.6 %) when compared to controls PLGA (26.0 ± 1.0 %) or saline (26.7 ± 1.7%). As expected, the treatment with soluble OVA did not render an increase in the percentage of specific asymmetric IgG (29.9 ± 2.5%). Nevertheless, no differences in the levels of specific asymmetric IgG were found between mice injected with PLGA or saline (day 85) and mice before treatment (day 50) (Figure 1).

Levels of OVA-specific IgG1, IgG2a and IgE. The effect of the desensitising therapy on the humoral Th2 and Th1 response was then evaluated. As shown

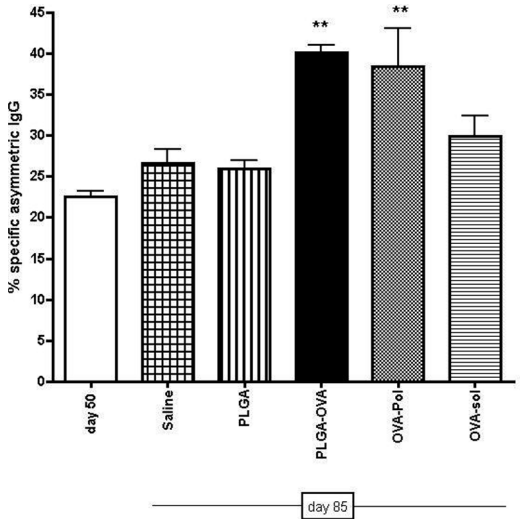

Figure 1: Specific asymmetric IgG serum levels in sensitised and desensitised mice. Levels of specific asymmetric IgG in sera from mice before (day 50) and after (day 85) the specific immunotherapy (SIT) determined by Concanavalin A-Sepharose affinity chromatography and ELISA. Each bar represents the mean ± standard error of individual sera, n = 5 animals per group. Data are representative of 3 independent experiments, **p < 0.01 vs day 50, saline or PLGA.

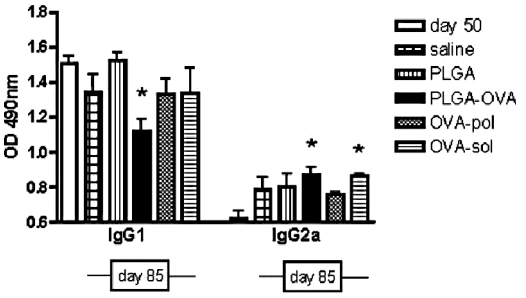

Figure 2: Specific IgG2a and IgG1 serum levels induced by different immunotherapies. Specific anti-OVA antibodies: IgG1 (left) and IgG2a (right) in mice sera before (day 50) and after (day 85) the SIT. Each bar represents the mean ± standard error of individual sera, n = 5 animals per group. Data are representative of 2 independent experiments, * $p < 0.05$ vs day 50.

in Figure 2, specific anti-OVA IgG1 levels were significantly reduced in the group of mice desensitised with PLGA-OVA and a concomitant increase in IgG2a level was observed. This may suggest a shift in antigen-specific lymphocytes from a Th2-type to a Th1-type response.

The desensitisation with OVA-pol did not modify either the levels of specific IgG1 or those of IgG2a. Upon analysing the results obtained with the SIT employing OVA sol, an increase in the serum levels of specific IgG2a was observed. The latter response was not accompanied by a significant decrease in the anti-OVA IgG1 levels (Figure 2).

The IgG1/IgG2a ratio of mice after SIT with PLGA-OVA was lower compared to IgG1/IgG2a ratio in mice before SIT (day 50), and when it was compared to mice treated with PLGA . This effect was not observed in sera of animals desensitised with OVA-sol or OVA-pol (Table 1).Our results indicate that no significant decrease in the levels of anti-OVA IgE was observed in any of the groups undergoing SIT (Figure 3).

Table 1: Specific ant-OVA IgG2a and IgG1 serum levels (expressed as OD 490 nm) induced by different immunotherapies.

	IgG1 (OD_{490nm})	IgG2a (OD_{490nm})	IgG1/IgG2a
Day 0	0.011 ± 0.003	0.054 ± 0.005	–
Day 50	1.506 ± 0.044	0.625 ± 0.039	2.4
Day 85			
Saline	1.343 ± 0.099	0.789 ± 0.071	1.7
PLGA	1.525 ± 0.043	0.800 ± 0.080	1.9
PLGA-OVA	1.118 ± 0.067 *,a	0.869 ± 0.043*,a	1.3*,a
OVA-pol	1.329 ± 0.092	0.758 ± 0.011	1.7
OVA-sol	1.338 ± 0.145	0.863 ± 0.017*	1.6

Specific anti-OVA antibodies: IgG1, IgG2a and the ratio IgG1/IgG2a before (day 50) and after (day 85) the SIT. Values are means ± standard error of individual sera, n = 5 animals per group. *$p < 0.05$ vs day 50, $^{a}p < 0.05$ vs PLGA.

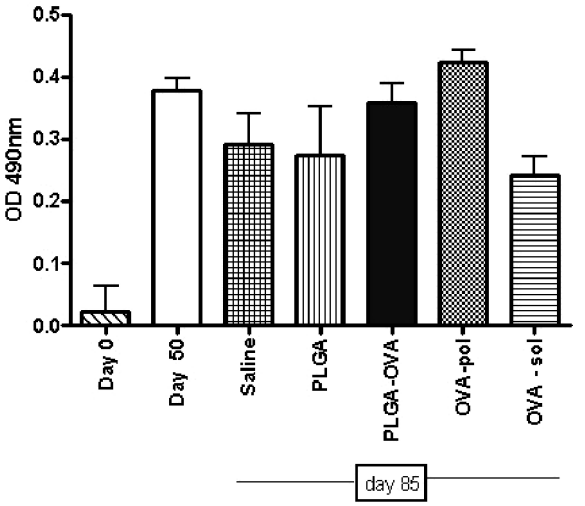

Figure 3: Specific IgE serum levels induced by different immunotherapies. Specific anti-OVA antibodies in mice sera before (day 50) and after (day 85) the SIT. Each bar represents the mean ± standard error of individual sera, n = 5 animals per group. Data are representative of 2 independent experiments.

PCA Test. It was also interesting to evaluate the protective effect of the asymmetric antibodies present in sera of animals desensitised with PLGA-OVA, sera which also presented a marked deviation towards a Th1 profile. A marked decrease in the reactivity in the PCA test was observed in sera taken at day 85 in the PLGA-OVA treated group (graded Mild + = 3–4 mm and low blue spot) when compared to sera taken at day 50 (non-desensitised mice: graded High +++> 10 mm and intense blue spot). This reduction in the PCA reactivity was partially reverted when serum was pre-adsorbed with Concanavalin A and therefore depleted of blocking asymmetric IgG (graded Mild + = 3–4 mm and low blue spot vs Moderate ++ = 5–10 mm and moderate blue intensity). Sera of animals treated with OVA-sol or OVA-pol showed moderate reactivity (graded Moderate ++ = 5–10 mm and moderate blue intensity) in the PCA test (Table 2).

Cytokine Levels in Splenocyte Culture Supernatants. Regarding the levels of IL-10 and IFNγ our finding show that splenocytes from mice treated with PLGA-OVA or OVA sol released higher levels of IL-10 and IFNγ when they were cultured in the presence of OVA in comparison with splenocytes from control mice treated with saline or PLGA (Figure 4). However, under our experimental conditions, IL-10 production by splenocytes from mice treated with OVA-pol was not modified. Although not statistically significant, IL-4 levels were lower in splenocytes culture supernatants from mice treated with PLGA-OVA compared to control PLGA (Figure 4).

Table 2: Cutaneous anaphylaxis test.

	Score
Day 0	–
Day 50	+++
Day 85	
PLGA-OVA	+
PLGA-OVA pre-adsorbed with Concanavalin A	++
OVA-sol	++
OVA-pol	++

PCA score obtained in mice inoculated with sera containing anti-OVA anaphylactic antibodies from sensitised mice (day 50) and after different treatments (day 85). n = 3 animals per group. A blue-coloured spot present in the antigen inoculation site >3 mm was considered a positive response: Mild + = 3–4 mm and low blue spot; Moderate ++ = 5–10 mm and moderate blue intensity; High +++ > 10 mm and intense blue spot.

DISCUSSION

Although the molecular mechanisms responsible for the healing process of IgE-mediated allergies induced by SITs are not quite clear, it has been accepted that the synthesis of allergen-specific IgG is of great importance in this process. This IgG has been named blocking IgG due to its capacity to interfere with the binding of the allergen to the IgE, thus blocking histamine release and passive cutaneous anaphylaxis (Bousquet, 1998; Ejrnaes, 2004; Wachholz, 2004). More recently, this concept of blocking antibody has been supplemented by evidence that IgG antibody–allergen complexes may inhibit mast cell signalling by cross-linking the immunoreceptor tyrosine activation motif (ITAM) -containing activating receptor FcεRI- with the immunoreceptor tyrosine inhibition motif (ITIM) -containing inhibitory receptor FcγRIIb- (Daeron, 1995; Strait, 2006). At present, we do not have any experimental data that could support this hypothesis in reference to the asymmetric IgG antibodies analyzed in the present study. Nevertheless, we consider it would be interesting to further analyze it in future studies.

Little is known about the role of asymmetric IgG antibodies in allergen-specific immunotherapy. In this report we studied the effect of different desensitising therapies in a murine model of allergy in relation to the modulation of the immune response induced by a particulate antigen. Levels of specific IgE, IgG1, and the cutaneous anaphylaxis test were employed to monitor the allergic status of sensitised mice (day 50). These animals were then subjected to different SITs employing microspheres of PLGA-OVA, OVA- pol (as model of particulate antigen to induce the synthesis of asymmetric antibodies) and OVA-sol (non-particulate antigen).

In the present study, we demonstrated for the first time an increase of specific IgG asymmetric antibodies induced by a SIT employing particulate

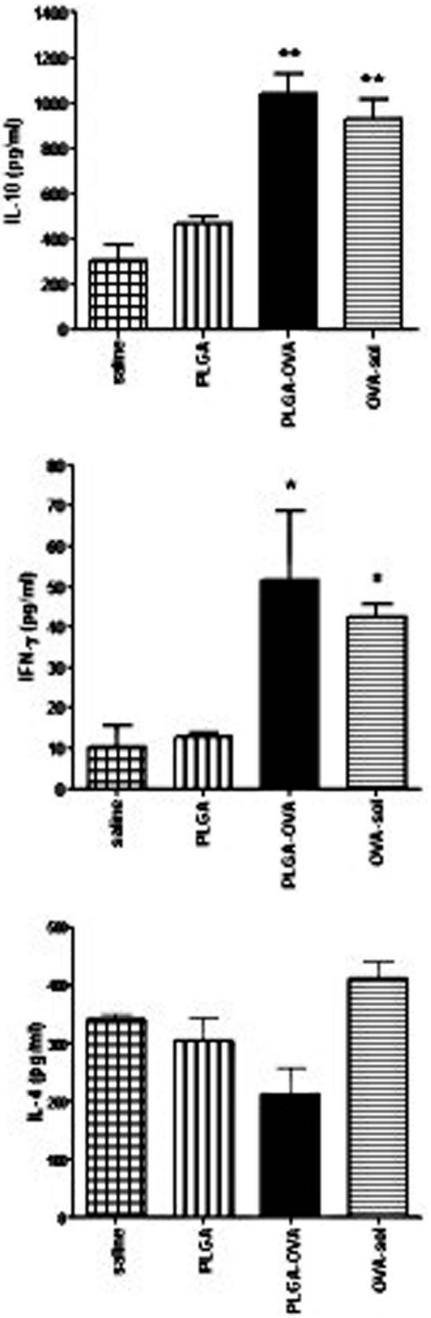

Figure 4: IL-10, IFNγ and IL-4 production in the spleen cells of PLGA-OVA and OVA-sol desensitised mice. Levels of IL-10 (upper panel), IFNγ (middle panel) and IL-4 (lower panel) determined by ELISA in culture supernatants of splenocytes mice receiving different SITs and then stimulated *in vitro* with OVA. Each bar represents the mean±standard error of quadruplicate cultures. Data are representative of 2 independent experiments. *$p < 0.05$, **$p < 0.01$ vs control mice (saline or PLGA).

antigen. In the case of the animals treated with PLGA-OVA, this increase was accompanied by a decrease in the capacity to induce passive cutaneous anaphylaxis and by the capacity to deviate the Th2 allergic response. These results are in line with those reported by other authors who consider the deviation of the immune response towards a Th1 phenotype to be beneficial in allergy (Carcaboso, 2004; Scholl, 2004).

OVA-pol induced an increase of specific IgG asymmetric antibodies; the results of this SIT are in concordance with previous observations reported by our team (Margni, 1983, 1986). However, in our study we did not observe a deviation towards a Th1 immune response, as had been demonstrated by Hayglass (1991); this is probably due to the fact that the OVA-Pol immunization scheme used in the present study was different (doses and frequency).

PLGA was chosen in the desensitising therapy on the basis that these microspheres can induce the maturation of antigen presenting cells (APC), thus favouring the stimulation of T-cells. The variability of the size of particles is advantageous; it has been demonstrated that particles of PLGA having a diameter ≤ 10 μm are more easily taken up by APC than bigger particles; thus, the former is more effective in stimulating the immune system. Nevertheless, the administration of particles with a size >10 μm made up of slow degradation polymers could be used as boosting doses (Johansen, 2000).

On the other hand, many experimental studies have demonstrated the importance of the changes in the glycosilation pattern of proteins, especially immunoglobulins (Leibiger, 1995; Leoni, 1986; Morelli, 1993). These changes alter their biological and physicochemical properties. As mentioned before, 15-20% of serum IgG molecules are asymmetric and their synthesis has been proven to be stimulated by particulate antigens. These antibodies behave as antigen-blocking and univalent, thus preventing the activation of immune effector mechanisms; this phenomenon plays an important biological role (Canellada, 2002; Malan Borel, 1989, Margni, 2002).

Our studies undertaken in an allergy model have shown that the SIT employing a single dose of PLGA-OVA induced an increase in the proportion of asymmetric IgG molecules similar to that achieved with 3 doses of OVA-pol. OVO-sol did not induce an increase of specific IgG asymmetric antibodies. The development of particulate vaccines has increased over the last years mainly because they are easily taken up by APC and transported to secondary lymphoid organs allowing a permanent delivery of antigens due to the low degradation or clearance of the carrier particle. Interestingly, it has been observed that several doses of OVA-pol had to be employed to achieve the asymmetric IgG levels obtained with single doses of PLGA-OVA. Therefore, the use of microparticles of PLGA would be advantageous in desensitising therapies, avoiding the use of multiple administrations to achieve protection.

Taking into account the differences observed when soluble or particulate antigens are used, it is possible that different presenting cells participate in antigen capture. Thus, cells could synthesize different factors (cytokines) that inhibit or stimulate the activity of glycosyl transferases, and these change glycosylation and produce different glycoforms. Alternatively, if soluble and particulate antigens are recognised by the same cell, then their capture by endocytosis (soluble antigen) or phagocytosis (particulate antigen) may activate different internal systems which induce modifications in the normal pattern of IgG glycosylation. Changes in glycosylation could occur through variations in the H chain peptide folding, which cause the exposure of previously hidden glycosylation sequons to glycosyltrasferase resulting in the increase of asymmetric antibodies (Miranda, 2005).

In order to evaluate the protective effect of asymmetric antibodies in allergy processes, we carried out the PCA test in mice belonging to the same strain. Considering that both murine IgG1 and IgE are homocytotropic, the protocol employed allowed us to evaluate the anaphylactic properties of IgG1, since antibodies of this isotype dissociate from cell surfaces more easily than IgE (after 2 and 48h, respectively) (Dearman, 2003). Serum from mice desensitised with PLGA-OVA displayed less activity in PCA tests; this reactivity was partially recovered when serum samples were pre-adsorbed with Con A-Sepharose and depleted of asymmetric IgG molecules. These results are in agreement with our previous studies demonstrating that asymmetric antibodies isolated from sera could bring about a PCA reaction, although with less efficiency than symmetric antibodies (Margni, 1972). The increase in the proportion of asymmetric IgG during a desensitising therapy would contribute to preventing the allergen from binding the specific IgE on mast cells and basophiles, thus leading to the reduction of anaphylactic reactions mediated by IgE.

Upon analysing the serum samples of mice before and after SIT it was observed that only those animals treated with one dose of PLGA-OVA displayed a decrease in the IgG1/IgG2a ratio when compared with the control group, mainly due to a decrease in the OVA-specific IgG1 and an increase in the serum specific IgG2a. These results are in line with those obtained by other authors who demonstrated that the use of allergen-loaded PLGA nanoparticles induce downregulation of an ongoing Th2 response in mice (Scholl, 2004).

Our results suggest that the deviation of immune response induced by PLGA-OVA towards the synthesis of anti-allergen asymmetric IgG and together with a change in the IgG1/IgG2a ratio would help diminish the anaphylactic reaction, as assessed by the PCA test even without a decrease in the specific IgE levels. Furthermore, it has been demonstrated that in humans, SITs do not produce a decrease in the IgE levels but an increase in the synthesis of blocking IgG4, which is associated with the control of the allergic reaction (Jarolin, 1990).

A significant increase in the levels of IL-10 was observed in splenocytes culture supernatants of animals treated with one dose of PLGA-OVA and 3 doses of OVA-sol when compared to non-desensitised control mice treated with PLGA and saline, respectively. Under our experimental conditions, this effect was not observed in supernatants of cell cultures from animals treated with OVA-pol. Although the immunological mechanisms by which the SIT administered by the sc route produces an increase in the levels of IL-10 are not known, the increase in the synthesis of this cytokine by splenocytes from animals which received PLGA-OVA and OVA-sol could be associated to the induction of regulatory CD4+CD25+ T-cells. In previous reports other authors demonstrated that allergen specific CD4+CD25+ cells can be stimulated by the use of SIT. This stimulation leads to the secretion of IL-10 and TGFβ, thus modulating the Th2 response generated by the allergen and skewing the humoral response towards the synthesis of blocking antibodies (Ejrnaes, 2004; Akdis, 2006; Nouri-Aria, 2004).

The immune modulation achieved by the PLGA-OVA treatment was also demonstrated by the increase of IFNγ levels observed in splenocytes culture supernatants and by the levels of IL-4, which tended to decrease over time. Culture supernatants of splenocytes from OVA-sol treated mice also presented higher levels of INFγ, as was demonstrated by other authors (Carcaboso, 2004; Nouri-Aria, 2004).

In summary, in the present study we found that among the SITs analyzed, PLGA-OVA is the only therapy that presents the best efficacy and more parameters indicative of the success of a desensitising therapy in a murine model of allergy (the synthesis of asymmetric antibodies, the decrease in Th2 response and a significantly activated Th1 immune response). We believe these findings would be of importance when designing new vaccination strategies for clinical use.

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Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. TG and JD designed the study protocol; CA, ERR and DCh carried out the assays; CM performed the statistical analysis of results. All authors carried out the analyses and interpretation of the data, contributed to the preparation and revision of the manuscript and read and approved the final version.

REFERENCES

Akdis, M., Blaser, K., Akdis, C. A. (2006). T regulatory cells in allergy. *Chem Immunol Allergy* 91:159–173.

Apicella, C., Custidiano, A., Miranda, S., Novoa, L., Dokmetjian, J., Gentile, T. (2006). Differential macrophage modulation of asymmetric IgG antibody synthesis by soluble or particulate stimuli. *Immunol Lett* 103(2):177–185.

Batanero, E., Barral, P., Villalba, M., Rodriguez, R. (2002). Biodegradable poly (DL-lactide glycolide) microparticles as a vehicle for allergen-specific vaccines: a study performed with Ole e 1, the main allergen of olive pollen. *J Immunol Meth* 259(1–2):87–94.

Batanero, E., Barral, P., Villalba, M., Rodriguez, R. (2003). Encapsulation of Ole e 1 in biodegradable microparticles induces Th1 response in mice: a potential vaccine for allergy. *J Control Release* 92(3):395–398.

Bousquet, J., et al. (1998). Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *Allergy Clin Immunol* Oct; 102(4 Pt 1):558–562. Review.

Canellada, A., Färber, A., Zenclussen, A. C., et al. (2002) Interleukin regulation of asymmetric antibody synthesized by isolated placental B cells. *Am J Reprod Immunol* 48(4):275–282.

Carcaboso, A. M., Hernández, R. M., Igartua, M., Rosas, J. E., Patarroyo, M. E., Pedraz, J. L. (2004). Potent, long lasting systemic antibody levels and mixed Th1/Th2 immune response after nasal immunization with malaria antigen loaded PLGA microparticles. *Vaccine* 22(11–12):1423–1432.

Chen, L. J., Chiang, C. H., Yeh, M. K. (2002). The mechanism of PLA microparticle formation by water-in-oil-in-water solvent evaporation method. *J Microencapsul* 19(3):333–346.

Ciprandi, G., Marseglia, G. L., Tosca, M. A. (2006). Allergen-specific immunotherapy: an update on immunological mechanisms of action. *Monaldi Arch Chest Dis* 65(1):34–37.

Coombes, A. G., Meikle, M. (1994). Resorbable synthetic polymers as replacements for bone graft. *Clin Mater* 17(1):35–67.

Daeron M., Malbec, O., Latour, S., Arock, M., Friedman, W. H. (1995). Regulation of high-affinity IgE receptor mediated mast cell activation by murine low affinity IgG receptors *J Clin Invest* 95:577–585.

Dearman, R. J., Skinner, R. A., Deakin, N., Shaw, D., Kimber, I. (2005). Evaluation of an in vitro method for the measurement of specific IgE antibody responses: the rat basophilic leukaemia (RBL). Cell Assay Toxicol 206(2):195–205

Dearman, R. J., Skinner, R. A., Herouet, C., Labay, K., Debruyne, E., Kimber, I. (2003). Induction of IgE antibody responses by protein allergens: inter-laboratory comparisons. *Food Chem Toxicol* 41(11):1509–1516.

Durham, S. R., Walker, S. M., Varga, E. M., et al. (1999). Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 12; 341(7):468–475.

Ejrnaes, A. M., Bodtger, U., Larsen, J. N., Svenson, M. (2004). The blocking activity of birch pollen-specific immunotherapy-induced IgG4 is not qualitatively superior to that of other IgG subclasses. *Mol Immunol* 41(5):471–478.

Finkelman, F. D., Rothenberg, M. E., Brandt, E. B., Morris, S. C., Strait, R. T. (2005). Molecular mechanisms of anaphylaxis: lessons from studies with murine models. *J Allergy Clin Immunol* 115(3):449–457.

Gehlhar, K., Schlaak, M., Becker, W. Bufe, A. (1999). Monitoring allergen immunotherapy of pollen-allergic patients: the ratio of allergen-specific IgG4 to IgG1 correlates with clinical outcome. *Clin Exp Allergy* 29(4):497–506.

Gentile, T., Malan Borel, I., Angelucci, J., Miranda, S., Margni, R. (1994). Preferential synthesis of asymmetric antibodies in rats immunized with paternal particulate antigen. Effect on pregnancy. *J Reprod Immunol* 22:173–183.

Guerin, V., Dubarryr, M., Robic, D., et al. (2002). Microsphere entrapped bee-venom phospholipase A2 retains specific IgE binding capacity: a possible use for oral specific immunotherapy. *J Microencapsul* Nov–Dec, 19(6):761–765.

Hayglass, K. T., Stefura, B. P. (1991). Anti-interferon gamma treatment blocks the ability of glutaraldehyde-polymerized allergens to inhibit specific IgE responses. *J Exp Med* 173(2):279–285.

Igartua, M., Hernandez, R. M., Gutierro, I., Gascon, A. R., Pedraz, J. L. (2001). Preliminary assessment of the immune response to Olea europaea pollen extracts encapsulated into PLGA microspheres. *Pharm Dev Technol* 6(4):621–627.

Jarolim, E., Poulsen, L. K., Stadler, B. M., et al. (1990). A long-term follow-up study of hyposensitization with immunoblotting. *J Allergy Clin Immunol* 85(6):996–1004.

Johansen, P., Men, Y., Merkle, H. P., Gander, B. (2000). Revisiting PLA/PLGA microspheres: an analysis of their potential in parenteral vaccination. *Eur J Pharm Biopharm* 50(1):129–146.

Nouri-Aria, K. T., Wachholz, P. A., Francis, J. N., et al. (2004). Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol* 172(5):3252–3259.

Labeta, M., Margni, R. A., Leoni, J., Binaghi, R. (1986). Structure of asymmetric non-precipitating antibody: presence of a carbohydrate residue in only one Fab region of the molecule. *Immunology* 57(2):311–317.

Leibiger, H., Hansen, A., Shoenherr, G., et al. (1995). Glycosylation analysis of polyreactive human monoclonal IgG antibody derived from a human-mouse heterohybridoma. *Mol Immunol* 32(8):595–602.

Leoni, J., Labeta, M., Margni, R. A. (1986). The asymmetric IgG non-precipitating antibody. Localization of the oligosaccharide involved, by concanavalin A interaction. *Mol Immunol* 23(12):1397–1400.

Malan Borel, I., Gentile, T., Angelucci, J., Binaghi, R., Margni, R. (1989). Asymmetrically glycosylated IgG isolated from non-immune human sera. *Biochim Biophys Acta* 990:162–164.

Malling, H. J. (1998). Immunotherapy as an effective tool in allergy treatment. *Allergy* 5:461–472.

Margni, R. A., Binaghi, R. (1972). Purification and properties of non precipitating rabbit antibodies. *Immunology* 22(4):557–563.

Margni, R. A., Gentile, T., Miranda, S. (2002). The antigen protective arm in a Th2-regulated immune response. *Inmunología* (Barcelona) 21:29–35.

Margni, R. A, Hajos, S. (1973). Biological and physicochemical properties of purified anti-DNP guinea pig non-precipitating antibodies. *Immunology* 24 (3): 435–443.

Margni, R. A, Malan Borel, I. (1998). Paradoxical behavior of asymmetric IgG antibodies. *Immunol Rev* 163:77–87.

Margni, R. A, Parma, A., Cerone, S., Erpelding, A., Perdigón, G. (1983). Agglutinating and non agglutination antibodies in rabbits inoculated with a particulate antigen (Salmonella thypimurium). *Immunology* 48(2):351–359.

Margni, R. A., Perdigón, G., Gentile, T., Abatángelo, C., Dokmetjian, J. (1986). IgG precipitating and co-precipitating antibodies in rabbits repeatedly injected with soluble and particulate antigens. *Vet Immunol Immunopathol* 13(1–2):51–61.

Miranda, S., Canellada, A., Gentile, T., Margni, R. (2005). Interleukin-6 and dexamethasone modulate in vitro asymmetric antibody synthesis and UDP-Glc glycoprotein glycosyltransferase activity. *J Reprod Immunol* 66: 141–150.

Morelli, L., Plotkin, L., Leoni, J., Fossati, C. A., Margni, R. A. (1993). Analysis of oligosaccharides involved in the asymmetrical glycosylation of IgG monoclonal antibodies. *Mol Immunol* 30(7):695–700.

Parma, A. E., Cerone, S., Erpelding, A., Margni, R. A. (1980). Immune response in rabbits inoculated with Salmonella typhimurium. *Rev Arg Microbiol* 12(3):100–104.

Postlethwait, R. W., Smith, B. M. (1975). A new synthetic absorbable suture. *Surg Gynecol Obstet* 140(3):377–380.

Roth-Walter, F., Schöll, I., Untersmayr, E., et al. (2005). Mucosal targeting of allergen-loaded microspheres by Aleuria aurantia lectin. *Vaccine* 23(21):2703–2710.

Scholl, I., Boltz-Nitulescu, G., Jensen-Jarolim, E. (2005). Review of novel particulate antigen delivery systems with special focus on treatment of type I allergy. *J Cntrl Rel* 104(1):1–27.

Scholl, I., Weissenbock,A., Forster-Waldl, E., et al. (2004). Allergen-loaded biodegradable poly (D,L-lactic-co-glycolic) acid nanoparticles down-regulate an ongoing Th2 response in the BALB/c mouse model. *Clin Exp Allergy* 34(2):315–321.

Sharif, S., Wheeler, A., O'Hagan, D. T. C. R. Society (Ed.) (1994). *Investigation of the Structural and Immunologic Integrity of an Allergen Entrapped in Poly(Lactide-Co-Glycolide) Microparticles*, Deerfield, Nice, France, 292–295.

Storni, T., Kundig, T. M., Senti, G., Johansen, P. (2005). Immunity in response to particulate antigen-delivery systems. *Adv Drug Deliv Rev* 57(3):333–355.

Strait, R., Morris, S., Finkelman, F. (2006). IgG-blocking antibodies inhibit IgE-mediated anaphylaxis in vivo through both antigen interception and FcγRIIb cross-linking. *J Clin Invest* 116(3):833–841.

Vila Jato, J. L. (1997). Aspectos fundamentales de los sistemas farmacéuticos y operaciones básicas. In: Tecnología Farmacéutica, Editorial Síntesis. Madrid, 75–142.

Wachholz, P. A., Durham, S. R. (2004). Mechanisms of immunotherapy: IgG revisited. *Curr Opin Allergy Clin Immunol* 4(4):313–318. Review.

Wise, D. L., Trantolo, D. J., Marino, R. T. (1987). Opportunities and challenges in the design of implantable biodegradable polymeric systems for the delivery of antimicrobial and vaccines. *Adv Drug Deliv Rev* 1:19–39.