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Characterization of Allergoids from Ovalbumin *in vitro* and *in vivo*

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Abstract

Several *in vivo* and *in vitro* methods for monitoring immunological properties of two allergoids obtained by formaldehyde treatment of ovalbumin (OA) were developed. The calculated molecular weight of allergoids was 80 kD (OA-F1) and 165 kD (OA-F2), respectively. The allergenic activity *in vitro* of allergoids in mast-cell histamine release assay was 1000 times lower than of OA. Both allergoids showed reduced ability to induce passive cutaneous anaphylaxis in the Sprague-Dawley rats or systemic anaphylaxis in Dunkin-Harley guinea-pigs. The ability of OA and allergoids to bind to the OA-specific IgE antibodies was measured *in vivo* by the inhibition of passive cutaneous anaphylaxis (PCA-inhibition). Allergoid binding to IgE was 51–66% lower than the native allergen. Moreover, the avidity of OA-specific IgG antibodies, measured by ELISA-inhibition, for allergoids and allergen was of the same order. Allergoids induced a different pattern of humoral immune response from that, induced by the native allergen. Thus, after immunization of BALB/c mouse, both allergoids induced a higher production of IgG and a lower production of IgE than OA, only OA-F2 induced a lower production of IgG₁. The differences in the IgA response to the immunogens was not significant. Delayed hypersensitivity studies in the BALB/c mouse showed that allergoids were 5- to 12-times less effective in inducing a cell-mediated immune response than OA. The present study provides a battery of immunological methods for preclinical testing of modified allergens.

Introduction

The search for isotype-specific regulators of IgE responses commands attention since these responses form the basis of allergic disease of the immediate type [1]. Immunotherapy of allergic disorders with gradually increasing doses of allergen

Abbreviations: BSA = bovine serum albumin; DTH = delayed type hypersensitivity; ELISA = enzyme-linked immunosorbent assay; IMM = isolation mast cell medium; MW = molecular weight; OA = ovalbumin; OA-F1 = allergoid from ovalbumin (80 kD); OA-F2 = allergoid from ovalbumin (165 kD); PBS = phosphate buffered saline; PCA = passive cutaneous anaphylaxis

extracts has been widely used and its efficacy and specificity has been proved in several controlled clinical studies. However, one of the main problems in the specific immunotherapy of atopic patients with native allergens is the appearance of local and systemic anaphylactic reactions at very low doses [2].

A rational approach to improving immunotherapy would be to use allergoids. The term 'allergoid' refers to a product that has much lower allergenic reactivity than the native allergen from which it was derived, while retaining the ability to induce allergen-neutralizing blocking antibodies [3, 4, 5, 6, 7,].

For the screening of the capacity of modified allergens to regulate IgE response a battery of preclinical assays is essential [8, 9, 10,]. It is thus important to verify whether the allergenicity of the allergoid is lower and whether its immunogenicity remains or increases in respect to that of native allergen, maintaining its therapeutic effect upon allergic manifestations [11, 12, 13].

As some studies indicate a relation between increased molecular mass and efficacy [14], we obtained two high molecular-weight allergoids by formaldehyde treatment of ovalbumin (OA). After physicochemical characterization, we examined their immunological properties in respect to native allergen to test allergenic activity, avidity for IgE and IgG and humoral and cellular immune response of allergoids. For this reason, we applied the following methods *in vivo* with slight modifications (PCA), humoral immune response in mice, delayed type hypersensitivity in mice). We also developed the following methods *in vitro* (histamine release assay, ELISA-inhibition) and *in vivo* (PCA-inhibition, anaphylactic shock in guinea pigs).

The findings should provide the basis for the use of these methods to characterize allergens and allergoids for immunotherapy.

Animals, Materials and Methods

Animals

Female Sprague-Dawley rats (240–350 g), BALB/c mice (20–22 g), and Dunking-Harley guinea-pigs (360–460 g) were purchased from Interfauna Iberica (Sant Feliu de Codines, Spain). They were fed on standard diet and kept in controlled climatic conditions. Food (UAR 04 for rats and mice and Bioure RMM for guinea-pigs) and drinking water were available *ad libitum*.

All animals were maintained and used in strict accordance with the guidelines issued by the EEC (86/609).

Preparation of formaldehyde-modified ovalbumin allergoids

OA (Fluka, Switzerland) was modified by treatment with formaldehyde following the method described by MARSH et al. [5]. The first allergoid (OA-F1) was obtained as follows: formaldehyde solution was slowly added to a solution of OA in 0.1 M phosphate-buffered saline pH 7.5 at 10 °C, with constant magnetic stirring. The pH of the solution was adjusted to 7.4–7.6 with NaOH 1N or HCl 1N, and the temperature was maintained at 10 °C. The final concentration of the reagents was adjusted to 2 mg/ml and 0.5 M for OA and formaldehyde, respectively. The mixture was incubated for 16 days at 10 °C and the pH was adjusted to 7.5. In subsequent step, the mixture was incubated for 16 days at 32 °C.

The second allergoid (OA-F2) was obtained with a similar procedure: first, formaldehyde and phosphate buffer were added to the OA solution to give 10 mg/ml OA, 2.5 M formaldehyde, pH 7.5 at 10° C. For the second incubation step, the solution was diluted five-fold and the procedure described above was followed.

The solutions obtained by the two procedures were extensively dialysed against PBS at 4° C to remove excess aldehyde.

The protein content was determined by the Biorad method, and the samples were stored at -20 °C until analysis.

Molecular weight (MW) distributions

The MW distributions of the allergen and allergoids were compared by analytical chromatography on Sephacryl S-300 (Pharmacia, Sweden). The column measured 1.5 × 90 cm and was equilibrated with 0.1 M phosphate buffer + 0.5 M NaCl + 0.02% NaN₃, pH 7.5, at room temperature. An upward flow of 15 ml/h was maintained by a peristaltic pump and 2.5 ml fractions were collected with an LKB automatic fraction collector under continuous UV monitoring at 280 nm (Pharmacia).

Before and after sample runs, the column was calibrated with the following markers: catalase (232 kD, Pharmacia), ferritin (440 kD, Pharmacia), thyroglobulin (669 kD, Pharmacia), aldolase (158 kD, Pharmacia), OA (43 kD, Fluka, Switzerland) and bovine serum albumin (67 kD, Sigma, USA).

Allergenic activity

a Histamine release assay

Mast cells were collected from the peritoneum of female Sprague-Dawley rats weighing 250–350 g. In brief, 20 ml of isolation mast cell medium (IMM) containing NaCl 150 mM, KCl 3.7 mM, Na₂HPO₄ × 2H₂O 3 mM, K₂PO₄ 3.5 mM, glucose 0.9 mM, BSA 0.1%, gelatin 0.1% and 10 U/ml heparin, were injected into the peritoneal cavity. The peritoneal fluid was collected and centrifuged for 15 min at 200 × g. The pelleted cells were suspended in IMM and passively sensitized with a 1 : 10 dilution of the mouse anti-OA serum for 1 h at 37 °C. Mast cells were washed and suspended in IMM at 1.33·10⁶ cells/ml. 300 µl of sensitized mast cell suspension was incubated for 10 min at 37° C. The reaction was induced with 100 µl of anti-IgE (1 : 10–1 : 200), OA, OA-F1 and OA-F2 (0.001–500 µg/ml) in IMM/Ca (0.9 mM Cl₂Ca) in the medium and maintained for 15 min at 37 °C. After the reaction the tubes were transferred into ice and centrifuged for 20 s at 15,000 × g. The supernatant and the sediment were separated and stored at -20° C until histamine assay. Histamine was measured by the fluorometric method described by SHORE [15]. The results are expressed as the percentage of histamine released.

b. Passive cutaneous anaphylaxis (PCA)

The capacity of OA and allergoids to bind mouse IgE antibodies to OA and to initiate a local anaphylactic reaction were compared by heterologous PCA on Sprague-Dawley rats (240–280 g). 0.1 ml of 1 : 80 dilution of serum from mice sensitized to OA was injected intradermally in duplicate into the shaved backs of rats. For the negative control, pooled serum from non-sensitized mice was used. 48 h later the rats received an intravenous injection of 2 mg of OA, OA-F1 in 1 ml 1% Evans blue dye in PBS. Thirty min later the rats were killed by anaesthetic overdose, the blue spots of the dorsal skin were removed and the dye was extracted by the procedure described by HARADA et al. [16]. The results are expressed as the µg of Evans blue extravasated.

c. Anaphylactic shock in guinea-pigs

Guinea-pigs were sensitized by subcutaneous injection of a primary dose of 5 mg/kg of OA (suspension containing 2 mg OA plus 400 mg Al (OH)₃ in 1 ml PBS) and an identical secondary dose 14 days later. Twenty-two days after the first dose, the animals received an intravenous challenge with increasing doses of OA (0.01–5 mg/kg), OA-F1 or OA-F2 (5–10 mg/kg) in PBS. Results are expressed as the minimum dose of OA and allergoids giving a lethal effect.

Avidity for IgE and IgG

a. Inhibition of passive cutaneous anaphylaxis (PCA-inhibition)

The capacity of OA and allergoids to inhibit the binding of OA to mouse IgE antibodies and to prevent a local anaphylactic reaction were compared by a variation of the PCA method. Prior the intradermal administration, the serum from sensitized mice was incubated with five-fold serial dilutions from 1250 to 10 µg/ml of OA, OA-F1 or OA-F2 overnight at 4 °C. The subsequent steps were as described above. The results are expressed as µg of the Evans blue extravasated by OA.

b. Avidity of specific IgG by ELISA-inhibition

The capacity of OA and allergoids to bind rat IgG antibodies to OA was compared by ELISA-inhibition. This method is a variation of an ELISA described previously [17]. In short, 1 : 80 dilutions of serum from OA-sensitized rats [18] was incubated overnight at 4 °C with 0.4, 2, 10 and 50 µg/ml of OA, OA-F1 or OA-F2. 100 µl of each neutralized serum was added in duplicate to micro ELISA plates (Labsystems, Spain) previously coated with 1.25 µg/ml of OA and incubated for 1 h at room temperature. The plates were washed and 100 µl of rabbit anti-rat IgG-Peroxidase (ICN-Biomedicals, Canada) was added and overnight at 4 °C. The assay was developed with 3-dimethylaminobenzoic acid (DMAB) and 3-methyl-2-benzothiazolinonehydrazone (MBTH) (Sigma-Aldrich Química, Spain) and the optical density was measured at 600 nm using a Titertek Uniskan microplate reader. The results are expressed as percentage inhibition of the binding of specific IgG to solid-phase by OA or allergoids.

Induction of immune responses

a. Humoral immune response in mice

The capacity of OA and allergoids to induce an antibody response were compared by immunization of BALB/c mice and determination of IgE, IgA, IgG and IgG₁ serum levels.

Mice were sensitized by intraperitoneal injection of a primary dose of 0.5 mg/kg of OA, OA-F1 or OA-F2 (suspension containing 50 µg of antigen plus 20 mg Al(OH)₃ in 1 ml PBS) and identical secondary dose 30 days later. The animals were bled 40 days after immunization and serum samples were stored at -20 °C until used for antibody titration.

Anti-OAS IgE serum levels were determined by PCA. In brief, 0.1 ml of a 1 : 80 serum dilution from sensitized mice was injected intradermally in duplicate into the shaved backs of Sprague-Dawley rats. 48 h later the rats received an intravenous injection of 10 mg of OA in 1 ml 1% Evans blue dye in PBS. Subsequent steps were performed as described above.

An ELISA method was used to determine serum levels of IgA, IgG and IgG₁ antibodies. Briefly, the wells of polystyrene plates were coated with 1.25 µg/ml of OA for IgG and IgG₁ and with 10 µg/ml of OA for IgA prepared in 0.15 M PBS, and incubated overnight at room temperature. Serially diluted samples were added and incubated for 1 h at room temperature. Bound antibodies were detected with peroxidase-labelled goat antibodies specific for murine isotypes (ICN-Biomedicals, Canada). Subsequent steps were as outlined above. Results are expressed as arbitrary units (U/ml) comparing from a standard serum with a given value of 50 U/ml for each IgA, IgG and IgG₁ anti-OA antibodies.

b. Delayed type hypersensitivity (DTH) in mice

BALB/c mice were sensitized by intraperitoneal injection of a primary dose of 0.5 mg/kg OA (suspension containing 50 µg OA plus 20 mg Al(OH)₃ in 1 ml PBS) and an identical secondary dose 30 days later. DTH was induced on day 40 after immunization. 10 µl of 2 mg/ml of OA, OA-F1 or OA-F2 in PBS was intradermally injected in the left ear. The right ear, injected with PBS, was used as control. 48 h later, the auricular ear edema was measured by weighing a circular section (7 mm diameter) of each ear. Results are expressed as percentage increase in the left ear weight.

Statistical evaluation

Mann-Whitney U test was used for the statistical evaluation of results.

Results

Molecular weight distributions

Figure 1 shows the peaks of the two formaldehyde preparations with respect to OA. The MW of allergoids was greater than OA. The calculated MW for OA-F1 was 80 kD, which suggests that it was formed by the condensation of two OA chains. The MW for OA-F2 was 165 kD and this was probably due to the condensation of 4 OA chains.

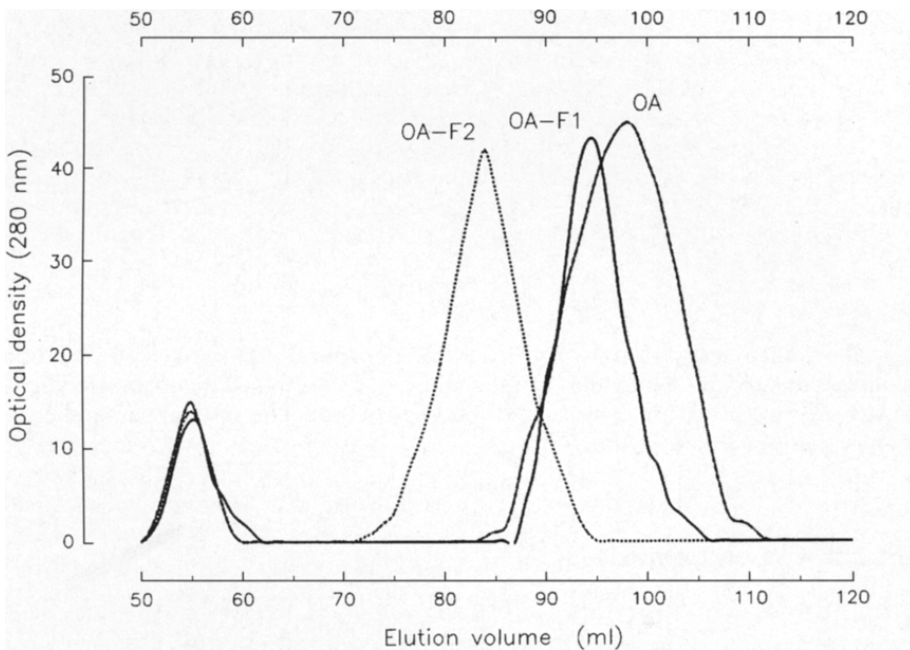


Figure 1. Chromatography of ovalbumin and the two allergoids (OA-F1 and OA-F2) on Sephacryl S-300 column (1.5 × 90 cm) equilibrated with 0.1 M phosphate buffer + 0.5 M NaCl + 0.02% NaN₃, pH 7.5. The allergoids were prepared by two-step formaldehyde modification of OA.

Allergenic activity by histamine release from mast-cells

Washed and sensitized mast-cells from rat peritoneum were incubated with a range of OA, OA-F1 and OA-F2 concentrations.

Before performing the histamine release assay, the presence of specific IgE antibodies on the mast-cells surface was tested by adding a range of anti-mouse IgE antibodies. A clear concentration-effect was always obtained, with a maximum histamine release of 32%.

The maximum histamine release produced by allergoids was about 20% with a dose of 100 µg/ml; the same histamine release was obtained using only 0.01 µg/ml of OA (Fig. 2).

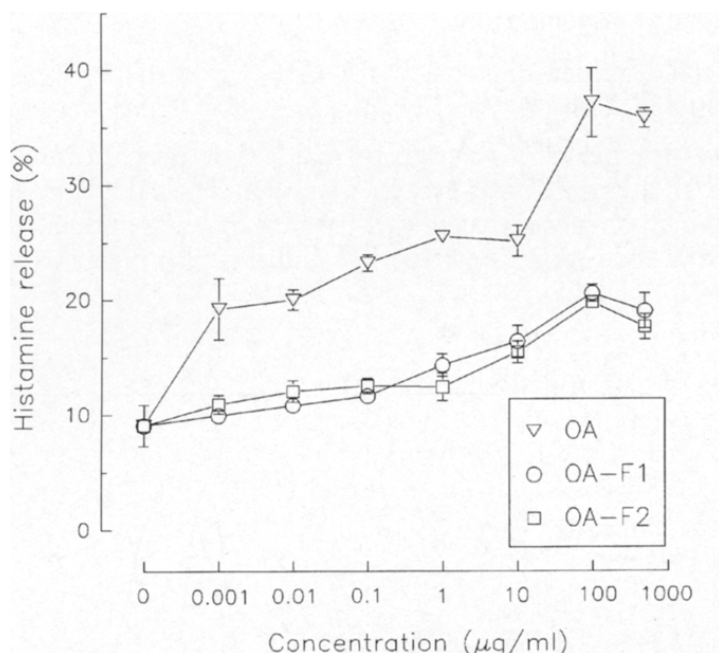


Figure 2. Histamine release from *in vitro* sensitized peritoneal rat mast-cell after addition of different amounts of OA and allergoids (OA-F1 and OA-F2) into cell incubations. The results are expressed as percentage release from total histamine content. The vertical bars indicate standard error of the mean of 4 determinations.

Passive cutaneous anaphylaxis (PCA)

Allergenic potencies of OA and allergoids to bind OA-specific IgE, studied by PCA, was measured. The maximum response was achieved by i. v. administration of OA, whereas OA-F1 and OA-F2 obtained only 7.2 and 20.8%, respectively, of the OA response ($p < 0.001$) (Fig. 3). Thus, the allergenicity of allergoids was reduced significantly compared to OA in this animal model.

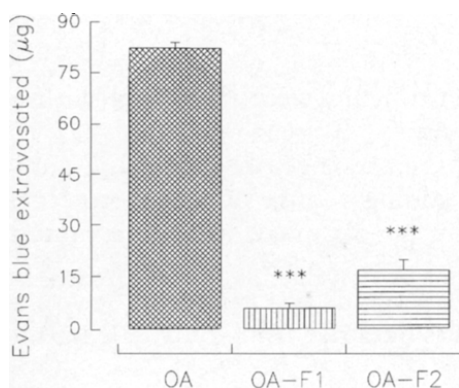


Figure 3. PCA assay comparing the capacities of OA, OA-F1 and OA-F2 to induce a local anaphylactic reaction after i. v. challenge with 10 mg/kg of antigen in rats passively cutaneous sensitized with 1 : 80 dilution of mice OA-specific serum. The results are expressed in µg of Evans blue extravasated. The vertical bars indicate standard error of the mean of 6–16 determinations. *** $p < 0.001$ Mann-Whitney U-test.

Anaphylactic shock in guinea-pigs

In OA-sensitized guinea-pigs, a lethal anaphylactic shock appeared in 90% of animals challenged with intravenous injection of OA at doses equal to or higher than 2 mg/kg. OA at 1 mg/kg induced signs of shock in all animals, half of them died from shock. Allergoids at 5 and 10 mg/kg failed to induce any sign of anaphylactic shock. The OA-sensitized animals that survived the administration of allergoids, died after receiving 5 mg/kg of OA.

Inhibition of passive cutaneous anaphylaxis (PCA-inhibition)

The capacities of OA and allergoids to inhibit the binding of OA-specific IgE to solid-phase OA are shown in Figure 4. Comparison of the mean inhibition values for OA-specific IgE showed that OA-F1 and OA-F2 at 1250 µg/ml were, respectively, 51% and 66% as effective in inhibiting Evans blue extravasation as OA at the same concentration. These allergenic activities (PCA and PCA-inhibition) revealed that OA-F1 and OA-F2 were substantially less allergenic than OA.

Avidity for specific IgG by ELISA-inhibition

The two allergoids resulting from formaldehyde modification were assayed for their capacities to inhibit the binding of OA-specific IgG to solid-phase OA. The degree of inhibition induced with allergoids and OA were similar. Thus, the IgG avidity for OA, OA-F1 and OA-F2 is the same (Fig. 5).

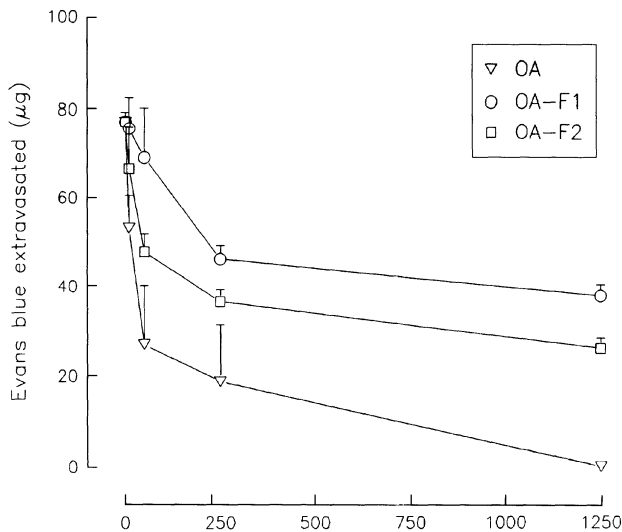
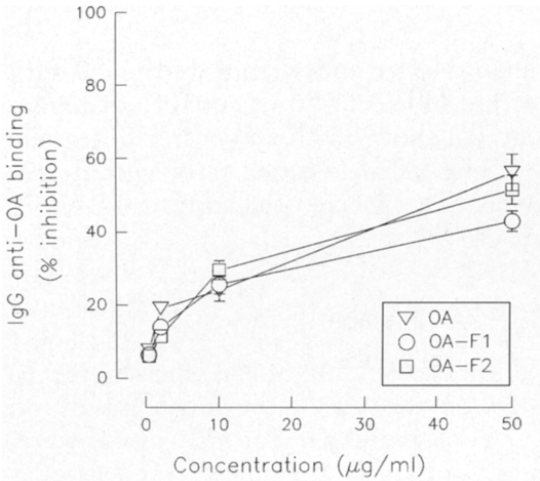
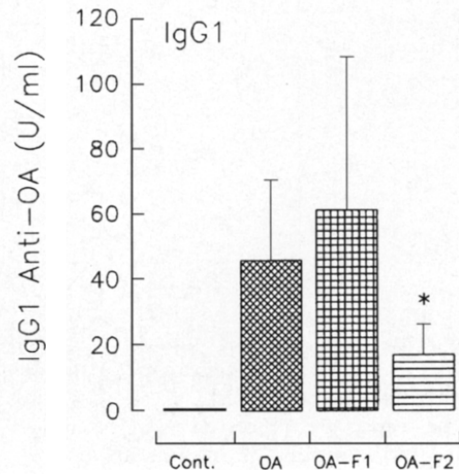
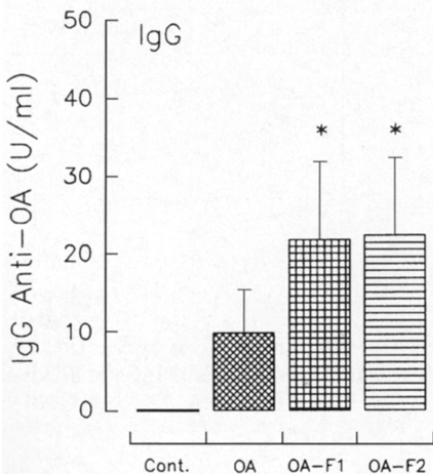
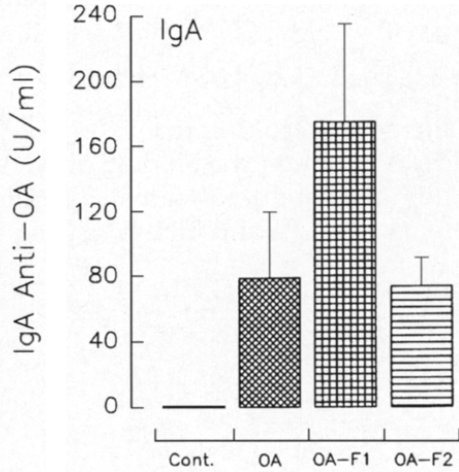
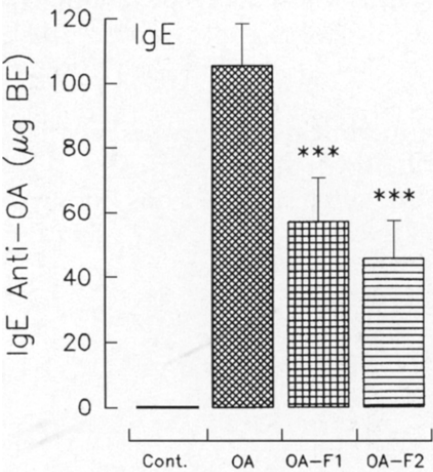


Figure 4. PCA-inhibition assay comparing the abilities of OA, OA-F1 and OA-F2 to prevent a local anaphylactic reaction induced by OA after incubation of 1 : 80 dilution of mice OA-specific serum with different amounts of OA, OA-F1 and OA-F2, previously to passive cutaneous sensitization. The results are expressed as µg of Evans blue extravasated. The vertical bars indicate standard error of the mean of 4 determinations.



↑

Figure 5. ELISA-inhibition assay comparing the abilities of OA and allergoids (OA-F1 and OA-F2) to inhibit binding of rat OA-specific IgG to solid phase OA. The results are expressed as percentage inhibition of binding by incubation of different amount of free allergen or allergoid with rat OA-specific 1 : 80 serum dilution. The vertical bars indicate standard error of the mean of 4 determinations.



Immunogenicity in mice

Figure 6 shows the OA-specific antibody levels after immunization of mice with OA, OA-F1 or OA-F2. The IgE levels obtained from OA-immunized mice were higher than those from OA-F1- and OA-F2-immunized mice. The allergenic capacity of allergoids was 50% lower than that of the allergen. These differences are highly significant ($p < 0.001$) in both cases with respect to OA. OA-F1 induced a higher degree of IgA immune response than OA, but the difference is not significant, whereas OA-F2 gave similar IgA levels to OA. In contrast, higher IgG levels were observed in allergoid-immunized mice than in OA-immunized mice ($p < 0.05$). The IgG₁ levels in OA-immunized mice were about 2-fold higher than the levels obtained with OA-F2 ($p < 0.05$). OA-F1 induced a higher IgG1 response than OA but the difference was not significant.

Induction of delayed type hypersensitivity (DTH) in mice

The capacity of OA and allergoids to induce hypersensitivity in OA-immunized mice are shown in Figure 7. The inflammatory response obtained by intradermal administration of OA-F1 and OA-F2 was 8% and 20% lower than the response induced by OA, respectively.

Figure 7. Delayed hypersensitivity response after intradermal injection of 20 μ g of OA, OA-F1 or OA-F2 in the left ear of OA-sensitized mice. The results are expressed as percentage increase of the left ear weight. The vertical bars indicate standard error of the mean of 5 animals. ** $p < 0.001$, * $p < 0.01$ Mann-Whitney U-test.

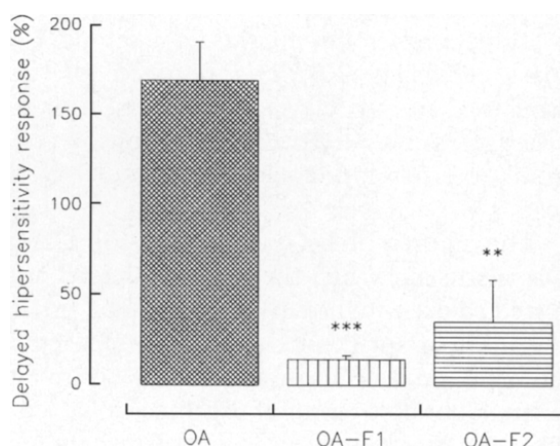


Figure 6. OA-specific IgE, IgA, IgG and IgG₁ antibody serum levels from BALB/c mice after intraperitoneally immunization with 0.5 mg/kg of OA, OA-F1 or OA-F2 (suspension containing 50 μ g of antigen plus 20 mg Al(OH)₃ in ml PBS) administered in days 0 and 30. Control group received PBS. Serum levels were measured after 40 days from primary dose. IgE antibody levels are expressed as median of μ g of Evans blue extravasated and IgA, IgG and IgG₁ as median of arbitrary units per ml. The vertical bars indicate the range of 7–10 animals. *** $p < 0.001$, * $p < 0.05$ Mann-Whitney U-test (see page 382).

Discussion

One of the claims for allergoids is their greatly reduced allergenic reactivity compared with the native allergen from which they were derived, while retaining other desirable properties characteristic of the native allergen. One of the main prerequisites for the potential use of allergoids in the immunotherapy of allergic diseases is that they should be able to be administered in high effective doses with negligible risk of systemic reactions [3, 4, 5].

The allergoids obtained by chemical modification of OA showed a higher molecular weight than the original allergen. The formaldehyde treatment was responsible for the production of polymeric structures for OA-F1 and OA-F2, due to its capacity to form intermolecular cross-linking [6]. The allergoids obtained were found, both *in vitro* and *in vivo* to have different immunological properties in respect to the native allergen.

It has been shown by all methods that allergoids had reduced allergenicity. *In vitro*, we found a higher reduction of allergenic activity of allergoids than *in vivo*. Thus, in mast-cell histamine release assay, a 1000-fold loss of allergenic activity was shown, whereas reductions in passive cutaneous anaphylaxis or systemic anaphylaxis of only to 16- to 30-fold were found *in vivo*. Data obtained from assays *in vitro* correlate well with histamine release assays performed on leukocytes with allergoids preparations [9, 11]. The development of this method on rat peritoneal mast cells suggested that the main source of histamine was from mast cells, since total histamine content was $25 \pm 5.9 \mu\text{g}/10^6$ mast cells, similar to data on isolated mast cells [19, 20]. The reduced allergenic capacity of allergoids could be explained in two ways: the reduction of the ability of the allergoid to bind specific IgE, as seen in the PCA-inhibition test, and a reduction in the ability of allergoids to cross-link two IgE molecules of the mast-cell surface as shown in PCA test.

The avidity of IgG antibodies for OA and allergoids was similar. This result was also seen when the avidity of IgM antibodies for allergen or allergoids was tested (data not shown). It should be born in mind that OA have multiple binding sites (or antigenic determinants), each of which is able to induce a differential isotypic response of antibodies in mice. These data seem to indicate that formaldehyde treatment produces in OA a selective modification that only affects epitopes that induce an IgE response in mice, without affecting IgG or IgM binding sites.

Besides the immunological reactivity, the aim of the present studies was to provide data to establish the safety of allergoids in the induction of systemic reactions. In our studies of systemic anaphylaxis in guinea-pigs, the administration of doses of allergoids five-to-ten fold higher than that of the allergen failed to produce systemic anaphylaxis. The finding that allergoids were unable to induce delayed hypersensitivity reactions in mice sensitized to the native allergen constitutes additional evidence that they are safe.

Allergoids retain the immunogenic properties of the OA, thus they are able to induce production of OA-specific IgG antibodies. The results showed that allergoids induce a higher response of IgG than OA. This result could be related with

the higher molecular weight of the allergoid [14]. In contrast, allergoids showed a poor capacity to induce an IgE antibody response to the native allergen.

The levels of IgG anti-OA are greater with allergoids, and their avidity for OA are similar, in contrast the IgE antibody response to allergoids is lower than with OA and the IgE anti-OA binding ability for allergoids is very low. Thus allergoids may be safer products than allergens and they may be more effective in treating allergic manifestations owing to production of a higher level of IgG blocking antibodies.

IgA secretory antibodies may be regarded as blocking antibodies that, when they are increased in immunotherapy, may produce beneficial actions such as inducing antiidiotypic responses which may suppress allergen-specific IgE antibody [4]. Moreover, a protective effect of IgA-specific antibodies on allergy was suggested by the increased incidence of atopic manifestations seen in children with IgA deficiency [21]. Our experimental data indicate that the IgA antibody response to allergoids does not differ from that induced by OA.

The efficacy of immunotherapy is usually measured by decrease in IgE antibody levels during immunotherapy, but measurement of the IgG₁ levels could also be interesting since both IgG₁ and IgE production were stimulated with IL-4 [22]. The modulation of T helper cell responses has a pivotal role in isotype-specific immunoglobulin responses. The activation of different T cell clones shows a biased pattern of cytokine secretion. IL-4 is produced by T_{H2} cells and has two major activities on activated B cells: stimulation of B-cells proliferation and a switch factor for IgE. T_{H1} cells are responsible for producing IFN- γ , which is related to IgG production, and in contrast, inhibits IL-4-induced IgE production. The IgG₁ response after immunization with OA-F1 was similar to that of OA. In contrast, after the immunization with OA-F2 the IgE and IgG₁ responses were lower than those observed with OA. So, IgE and IgG₁ response change in parallel with OA-F2 but not with OA-F1. The lower induction of IgE and IgG₁ and the increased IgG production after allergoid immunization suggest an allergoid-specific activation of T_{H1} cells. Further studies are needed on different allergens before these findings can be generalized. The problem is that allergenic epitopes of allergens for laboratory animals may not be allergenic for humans and these potential differences could make it difficult to establish an activity/safety profile of allergoids related to native allergen. The importance of different immunoglobulin responses after allergen/allergoid immunization will only become apparent after determining comparative therapeutic efficacy of allergoids.

In conclusion, the present studies provide an immunologic battery of tests for preclinical testing of modified-allergens activity and safety in laboratory animals previously to human administration.

References

1. ISHIZAKA, K. 1988. IgE binding factors and regulation of the IgE antibody response. *Ann. Rev. Immunol.* **6**: 513.
2. MALLING, H. J., and B. WEEKE. 1993. Position paper: Immunotherapy. *Allergy*. **48**: 9.

3. PUTTONEN, E., L. PILSTRÖM, J. WHAN, and H. J. MAASCH. 1982. Studies on allergen and allergoid preparations from purified timothy (*Phleum pratense*) pollen extracts. II. Anaphylaxis studies in rats and histamine release from human leucocytes. *Int. Archs. Allergy appl. Immunol.* **68**: 7.
4. WHEELER, A. W. 1990. Novel allergen forms for immunotherapy. Stockholm, Repro-Print AB, Stockholm, 43 pp.
5. MARSH, D. G., P. S. NORMAN, M. ROEBBER, and L. M. LICHTENSTEIN. 1987. Studies on allergoids from naturally occurring allergens. III. Preparation of ragweed pollen allergoids by aldehyde modification in two steps. *J. Allergy Clin. Immunol.* **68**: 449.
6. MAASCH, H. J., MARSH DG: Standardized extracts. Modified allergens-allergoids. *Clin. Rev. Allergy.* **5**: 89.
7. PUTTONEN, E., H. J. MAASCH, and L. PILSTRÖM. 1982. Studies on allergen and allergoid preparations from purified timothy (*Phleum pratense*) pollen extracts. I. Physiocochemical characteristics and binding to allergen-specific human IgE. *Int. Archs. Allergy appl. Immunol.* **68**: 1.
8. DREBORG, S. 1993. Standardization of allergenic preparations by *in vitro* and *in vivo* methods. *Allergy.* **48**: 63.
10. PATTERSON, R., I. M. SUSZKO, C. R. ZEISS, and J. J. PRUZANSKY. 1981. Development and evaluation of polymerized allergens for immunotherapy. *Int. Archs. Allergy appl. Immunol.* **66**: 293.
11. MAASCH, H. L., B. FISCHER, and R. WAHL. 1984. Comparison of histamine release assay and RAST inhibition as tools for allergen extract standardization. *Int. Archs. Allergy appl. Immunol.* **73**: 314.
12. SALDAGO, J., A. CAMPAMÁ, A. GILBERT, and J. QUERALT. 1992. Biological characterization of a formulated allergoid from *Dermatophagoides pteronyssinus*. *Rev. Esp. Fisiol.* **48**: 245.
13. PLATTS-MILLS, T. A. E. 1979. Local production of IgG, IgA and IgE antibodies in grass pollen hay fever. *J. Immunol.* **122**: 2218.
14. HAYGLASS, K. T., and G. H. STREJAN. 1984. Suppression of the IgE antibody response by glutaraldehyde-modified ovalbumin: dissociation between loss of antigenic reactivity and ability to induce suppression. *Int. Archs. Allergy appl. Immunol.* **74**: 332.
15. SHORE, P. A., A. BURKHALTER, and V. H. COHN. 1959. A method for the fluorometric assay of histamine in tissues. *J. Pharmacol. Exp. Ther.* **127**: 182.
16. HARADA, M., M. TAKEUCHI, T. FUKAO, and K. KATAGIRI. 1971. A simple method for the quantitative extraction of dey extravasated into the skin. *J. Pharm. Pharmacol.* **23**: 218.17.
- SALGADO, J., A. GILBERT, M. CASTELL, C. CASTELLOTE, and J. QUERALT. 1988. ELISA for quantification of specific IgG and IgE antibodies to ovalbumin. *Allergol. et Immunopathol.* **16**: 95.
18. SALDAGO, J., A. GILBERT, A. FRANCH, M. CASTELL, C. CASTELLOTE, and J. QUERALT. 1989. Effect of adjuvants on IgG and IgE response to ovalbumin in rats. *Allergol. et Immunopathol.* **17**: 237.
19. ENERBÄCK, L., and U. WINGREN. 1980. Histamine content of peritoneal and tissue mast cells from growing rats. *Histochemistry* **66**: 113.
20. STEVENS, R. L., and K. F. AUSTEN. 1989. Recent advances in the cellular and molecular biology of mast cells. *Immunol. today* **10**: 381.
21. LÚDVIKSSON, B. R., T. H. ERIKSSON, B. ARDAL, A. SIGFÚSSON, and H. VALDIMASSON. 1992. Correlation between serum immunoglobulin A concentrations and allergic manifestations in infants. *J. Pediatrics* **121**: 23.
22. SNAPPER, C. M., F. D. FINKELMANN, and W. E. PAUL. 1988. Regulation of IgG₁ and IgE production by interleukin 4. *Immunol. Rev.* **102**: 51.