

Monomeric chemically modified allergens: immunologic and physicochemical characterization

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Allergenic extracts (*Der p*, grass, and *Parietaria*) or single allergens such as *Par j* I (the major allergen of *Parietaria*) and ovalbumin (OA), a food allergen widely used in animal models, were chemically modified by reaction with potassium cyanate (KCNO), which transforms the ϵ -amino group of the lysine of proteinaceous allergens into ureido groups. KCNO-modified (carbamylated) allergens have low allergenic potency, as demonstrated *in vitro* (RAST inhibition) and *in vivo* (passive cutaneous anaphylaxis). When used to immunize rabbits, carbamylated allergens still induce IgG antibodies able to cross-react with native allergens (immunoblotting experiments). An interesting feature distinguishing carbamylated allergens from other chemically modified allergens is the preservation of the native monomeric dimension as demonstrated by SDS-PAGE analysis. Results are discussed from the perspective of clinical application of carbamylated allergens.

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Immunotherapy (IT) with subcutaneously injected allergenic extracts has been used for decades to treat allergies. The clinical benefits are well documented (23, 26), even though the mechanisms are still not completely defined. Though the protective role of the specific IgG response induced by IT correlates reasonably well with the clinical response in Hymenoptera allergy (5, 10, 15), it is still controversial in other allergic situations (6, 18). There are now reports of the induction of specific suppressor T-lymphocyte activity and interference with production of the cytokines involved in allergic phenomena (7, 28, 32).

The risk of sometimes severe side-effects associated with the injection of allergenic extracts (16) has led allergists to explore safer therapeutic modalities. Chemically modified allergens have been developed. These products, called “allergoids”, are allergens with reduced allergenic but fully preserved immunogenic properties (19). Polymerized allergoids obtained by reacting allergenic extracts with aldehydes have been tested in clinical trials (17).

The high molecular mass of these extracts presumably makes them unable to cross biologic membranes; therefore, they cannot be used to express any therapeutic effect by routes of administration

involving transmucosal passage. On the other hand, the rapid absorption of allergens after local administration (21, 23) suggests it would be safer to use allergenic extracts with lower allergenic potency for IT, as this would lower the risk of side-effects.

We have developed new, chemically modified allergens that, unlike allergens modified with aldehydes, retain their original monomeric nature. The chemical procedure, known as carbamylation (4, 30, 31), is based on the use of potassium cyanate in experimental conditions (8) that selectively promote substitution of the ϵ -amino group of the lysine moiety of proteins present in allergenic extracts or in single allergens such as *Par j* I and ovalbumin (OA).

Carbamylated allergens have been characterized for their biologic activity and physicochemical features and have potential for the therapeutic control of allergic diseases.

Material and methods

Extracts and single allergens

Dermatophagoides pteronyssinus (*Der p*) whole cultures and *Parietaria judaica* and grass pollens

(Entofit, Milan, Italy) were defatted with ether, and the residues were extracted (5% w/v) in 20 mM phosphate buffer for 24 h at 4°C, under stirring. Particulate material was removed by centrifugation (18 000 g for 30 min), and the supernatant was gel-filtered on a Sephadex® G-25 column. After calibration with blue dextran, fractions corresponding to the excluded peak were collected, pooled, and lyophilized.

Par j I was purified by affinity chromatography using specific monoclonal antibodies, as previously described (22).

The protein content of extracts and *Par j* I allergen was determined according to Bradford (3). Ovalbumin was purchased from Sigma (Milan, Italy) as powder.

Chemical modification

Lyophilized *Der p*, pollen extracts, *Par j* I (5 mg/ml), and OA (10 mg/ml) were dissolved in sodium tetraborate buffer 0.1 M, pH 9.2; freshly crystallized potassium cyanate (KCNO) was added to obtain a final concentration of 0.5 M. In blank experiments, potassium chloride (0.5 M) was added instead of KCNO. The resulting solutions, including a blank sample, were incubated at 40°C for 16 h; during the first hour, the solution was kept at pH 9.2 by addition of 0.1 M potassium hydroxide or phosphoric acid. The reaction was stopped by desalting on a Sephadex G-25 column. Fractions corresponding to the excluded peak were collected in 20 mM sodium phosphate buffer, pH 6.8, and stored at 4°C. The degree of substitution was checked by reacting the residual ε-amino group with 2,4,6-trinitrobenzene sulfonic acid (TNBS) according to Fields (9).

RAST-inhibition experiments

Modified products (allergenic extracts, *Par j* I and OA) were tested for allergenic potency, in parallel with nonmodified (native) products, by RAST inhibition according to Yman et al. (33), with some modifications. Briefly, 30 µl volumes of serial dilutions of native or modified extracts (*Der p*, *P. judaica* and grass pollens, *Par j* I, and OA) were mixed in tubes with 20 µl of a pool of sera from patients allergic to the pertinent allergen. Positive and negative controls consisted of 30 µl of phosphate-buffered saline (PBS) preincubated with either the same pool or a RAST-negative serum, respectively.

After a 2-h incubation, a polystyrene bead sensitized with the appropriate allergen (Sferikit®, Lab. Farmaceutico Lofarma, Milan, Italy) was added to each tube and incubated overnight. Bound IgE was detected with 50 µl of ¹²⁵I-labeled anti-IgE serum.

The residual radioactivity was measured in a gamma scintillation counter. Data were expressed as percentage of inhibition by the following formula:

% inhibition =

$$\frac{(\text{cpm positive control}) - (\text{cpm sample})}{(\text{cpm positive control}) - (\text{cpm negative control})} \times 100$$

and were plotted and evaluated according to the US Food and Drug Administration protocol (1). The volume of sample (as µl) inhibiting the RAST response by 50%, defined as C₅₀, was established and used to compare the allergenic potency of the various samples.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli (14) using a Protein II apparatus (Bio-Rad, Milan, Italy) with a discontinuous buffer system. Native or modified samples (OA and *Par j* I) were diluted in running buffer (5% β-ME) and boiled for 5 min before the run. Protein components were resolved on a 7% (OA) or a 10–20% gradient (*Par j* I) with a standard protein mixture as mol. wt. reference. Gels were stained with Coomassie brilliant blue R-250 to visualize the separated proteins.

Animals

Balb/c mice were used for production of IgE antibodies, Sprague-Dawley rats for passive cutaneous anaphylaxis, and New Zealand white rabbits for antiserum production. All animals were supplied by Charles River, Calco, Como, Italy.

Induction of IgE response in mice

Balb/c mice were given an i.p. injection of 10 µg (as protein content) of native (OA) or modified OA (m-OA), or 10 µg of native (grass) or modified grass extract (m-grass) in 0.25 ml saline with the addition of 1 mg Al(OH)₃. A third group of animals received only 0.25 ml of saline plus 1 mg Al(OH)₃. Twenty-eight days later, each animal was boosted with the same allergen as before. After a week, blood was collected, and sera from three mice of each group were pooled. The specific IgE titer was determined by mouse-rat passive cutaneous anaphylaxis (24).

Passive cutaneous anaphylaxis (PCA)

The capacity of native and modified allergens (OA and grass pollen extract) to induce a specific IgE response *in vivo* was evaluated by mouse-rat PCA assay.

Sprague-Dawley rats were passively sensitized by intradermal injection of 50 μ l of sera from mice treated as described before. Sera were diluted two-fold from 1/10 to 1/1280 for OA and from 1/2 to 1/1024 for grass extract. Each dilution was tested in triplicate in different animals. PCA reactions were elicited 48 h later by challenging these sensitized animals with an i.v. injection of 1 ml of saline containing 2 mg of OA or grass extract and Evans blue dye (0.5%). Rats were killed 30 min later, and the PCA titer was determined as the reciprocal of the highest dilution of the mouse serum giving a reaction 5 mm in diameter.

Antisera production

Antisera to native or modified *P. judaica* extract were raised in rabbits by subcutaneous injection of 1 ml of an emulsion of Freund's complete adjuvant containing 3 mg of native or modified proteins, every 2 weeks for 2 months. Freund's incomplete adjuvant was used for subsequent challenges. One week after the last injection, blood was taken, and serum was checked in immunoblotting experiments.

Immunoblotting experiments

P. judaica extract in Tris buffer containing 2% SDS was boiled for 5 min, and an electrophoretic run was done on a linear 15% polyacrylamide gel. Resolved proteins were transferred to nitrocellulose paper, which was then incubated with serum from normal rabbits or rabbits immunized with native and modified *P. judaica* extract. The signal was developed with a biotinylated serum antirabbit IgG (Sigma, Milan, Italy) and streptavidin peroxidase (SPA, Milan, Italy) with diaminobenzidine (Sigma, Milan, Italy) as substrate.

Statistical analysis

PCA titers were log transformed, and means $\pm$ SD were compared by the Mann-Whitney U test.

Results

Allergenic activity (human IgE binding) of native and modified allergens

The allergenic potency of native and modified allergens (*P. judaica* and grass pollen extracts, *Der p*, *Par j* I, or OA) was compared by RAST-inhibition experiments.

Table 1 shows the C_{50} values, which are inversely proportional to allergenic potency. For all allergens, the C_{50} of the modified form was significantly higher

Table 1. Allergenic potency of native and modified forms of allergens evaluated by RAST inhibition

Allergens	C_{50} values	
	Native	Modified
Grass	0.030 $\pm$ 0.003*	58.0 $\pm$ 15.5*
<i>P. judaica</i>	0.0032 $\pm$ 0.001	1.36 $\pm$ 0.1
<i>Der p</i>	0.046 $\pm$ 0.008	19.4 $\pm$ 02.9
Ovalbumin	0.0027	0.57
<i>Par j</i> I	0.0088 $\pm$ 0.009	2.27 $\pm$ 2.28

m $\pm$ SD = mean $\pm$ standard deviation.

C_{50} = volume of allergen (μ l) needed to inhibit response by 50%.

than for the native form, indicating that the chemical reaction with KCNO considerably reduced their allergenic potency.

No difference was found between blank and native samples (data not shown).

In vivo allergenicity of native and modified allergens

The modified allergens (OA and grass extract) induced significantly less specific IgE response *in vivo*, measured as PCA assay, than the native allergens. As shown in Table 2, the PCA titers determined with pooled sera were 2.8 (OA) and 2.3 (grass extract) for the native and 1.7 and 1.15 for the modified forms. These data agree with *in vitro* findings (RAST inhibition), confirming the dim-

Table 2. Evaluation of *in vivo* allergenicity of native and modified allergenes

Groups	Log ₁₀ PCA titer			
	1-3 mice	4-6 mice	7-9 mice	m $\pm$ SD
Control	<1.0	<1.0	<1.0	—
Native OA	3.1	2.8	2.5	2.8 $\pm$ 0.2*
	3.1	2.8	2.8	
	2.8	2.5	2.8	
Modified OA	2.2	2.2	1.6	1.7 $\pm$ 0.3
	1.9	1.6	1.3	
	1.9	1.6	1.6	

Groups	Log ₁₀ PCA titer			
	1-3 mice	4-6 mice	—	m $\pm$ SD
Control	<0.9	<0.9	—	—
Native-grass extract	2.4	2.1	—	2.3 $\pm$ 0.15*
	2.1	2.4	—	
	2.4	2.4	—	
Modified-grass extract	1.5	0.9	—	1.15 $\pm$ 0.22
	1.2	0.9	—	
	1.2	1.2	—	

m $\pm$ SD = mean $\pm$ standard deviation.

* P < 0.01 between native and modified forms (OA and grass).

inished allergenic potential of these modified molecules.

The PCA and RAST-inhibition results and the ratio of native to modified group show a clear difference between PCA titer (12.8 for OA/m-OA and 14.1 for grass/m-grass) and RAST C_{50} values (211 and 1933, respectively).

Immunogenicity of native and modified allergens

For evaluation of their capacity to induce an IgG response, rabbit hyperimmune sera obtained by

immunization with native or modified *P. judaica* allergens were tested in immunoblotting experiments (Fig. 1). Sera from rabbits immunized with the modified *P. judaica* extract showed a pattern of antigen recognition (including *Par j I* allergen identified on the basis of molecular mass) very similar to that of sera from rabbits immunized with the nonmodified allergen, supporting the theory that modified extract preserves the capacity *in vivo* to induce an IgG response, cross-reacting with the components present in native extract.

Mol. mass

kDa

67

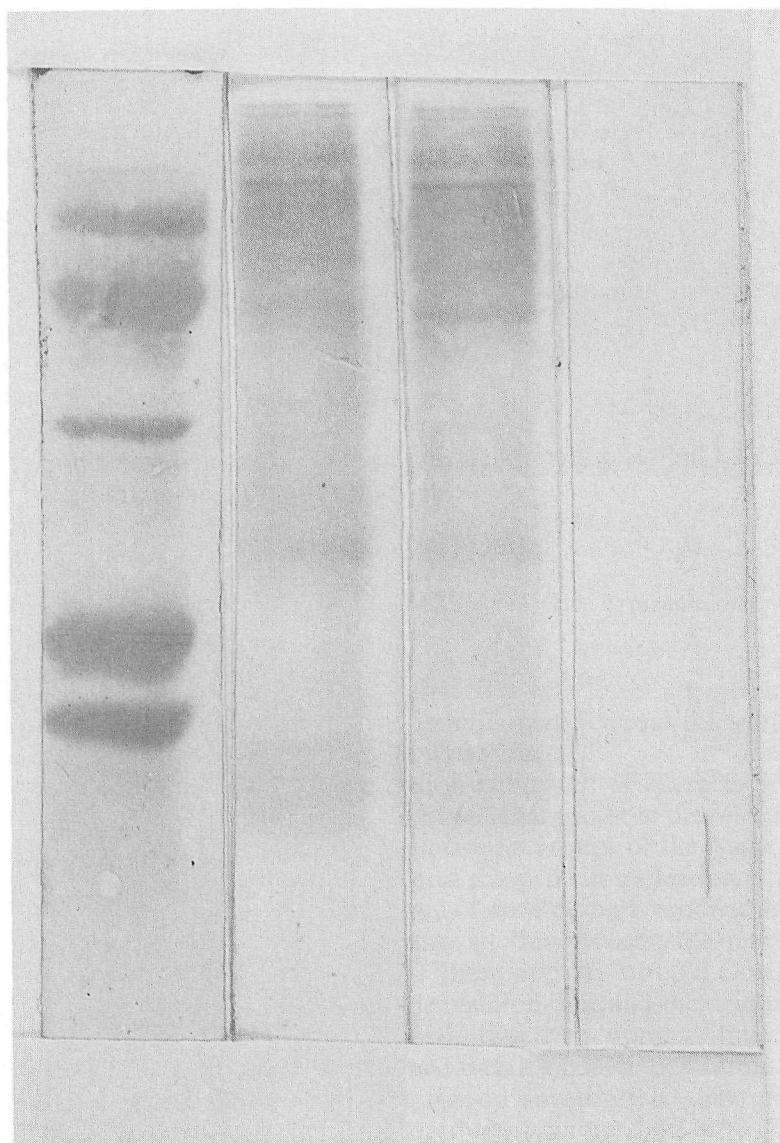
43

30

17

13.5

Par j I



M

1

2

3

Fig. 1. Immunoblotting experiments of SDS-PAGE *P. judaica* resolved proteins. Lane M) molecular mass markers; lane 1, serum from rabbit immunized with native *P. judaica* extract; lane 2) serum from rabbit immunized with modified *P. judaica* extract; lane 3) normal rabbit serum.

SDS-PAGE characterization of native and modified allergens

Carbamylation by KCNO is based on the transformation of ϵ -amino groups of the lysine moiety present on the proteins into ureido groups. The degree of NH_2 -group substitution is a valuable marker of the modification. In the modified allergenic extracts, as in single allergens, this was more than 80% (data not shown).

When analyzed by SDS-PAGE (Figs. 2 and 3), both allergens (OA and *Par j* I) as native and modified forms showed bands at the same molecular

mass (about 43 kDa for OA and about 12.5 kDa for *Par j* I), confirming that the reaction with KCNO, while reducing the allergenic potency (Table 1), does not influence the size of the modified molecule, which thus maintains the monomeric nature of the original one.

Discussion

Despite the proven efficacy of IT, its potential for inducing systemic allergic reactions has limited its widespread use. Efforts to improve it have been made in various directions. The "depot" formula-

Mol. mass

kDa

94

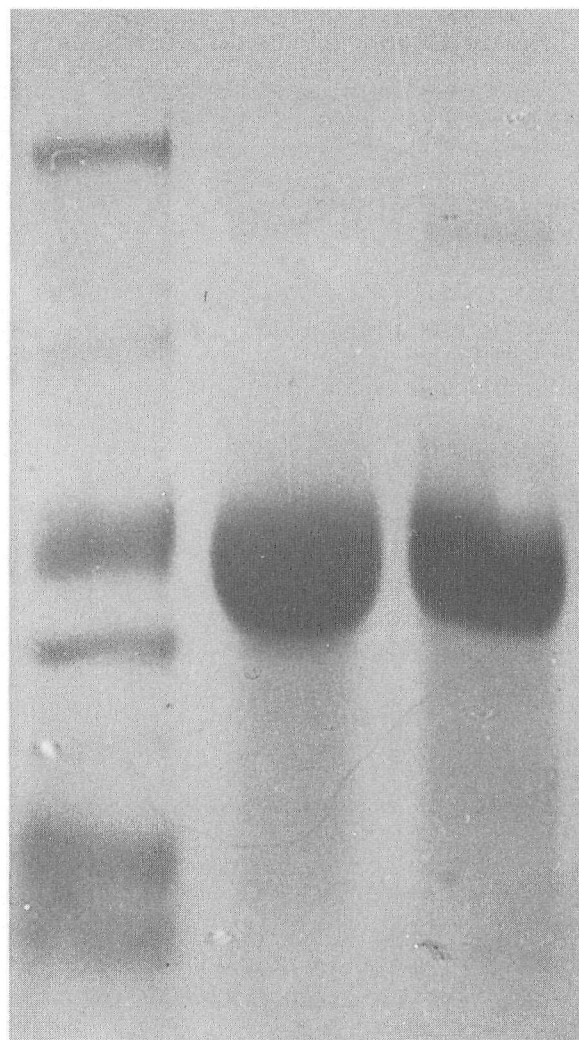
67

43

30

17

13.5



M

1

2

Fig. 2. SDS-PAGE analysis of native and modified OA. Lane M) molecular mass markers; lane 1) native OA (2 mg/ml); lane 2) modified OA (2 mg/ml).

Mol. mass

kDa

94 —

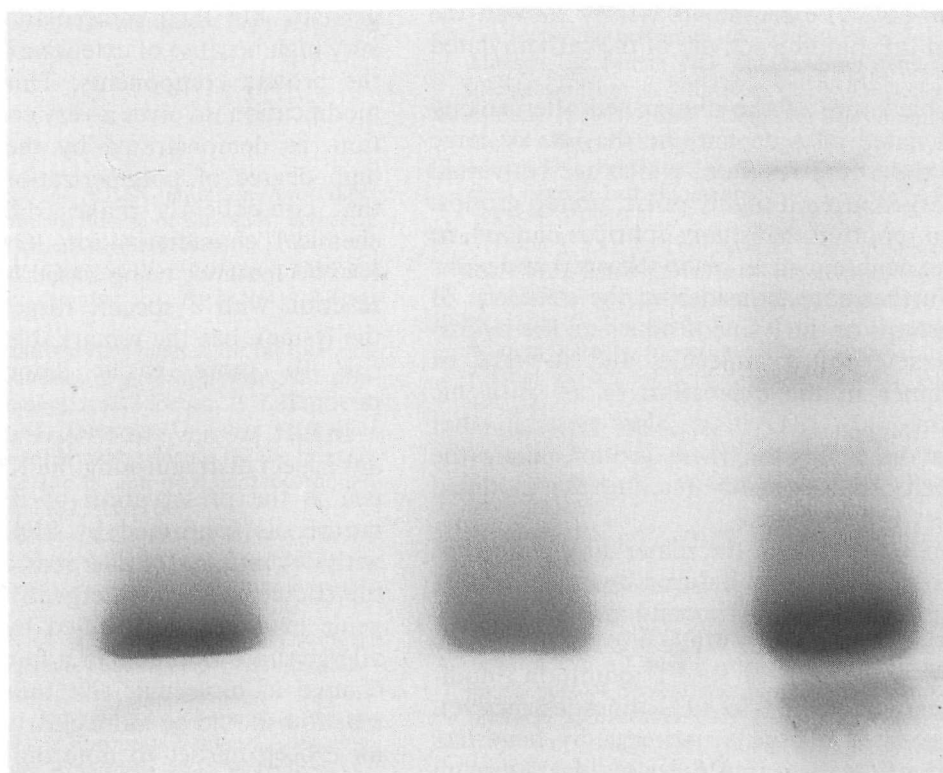
67 —

43 —

30 —

17 —

13.5 —



A

B

C

Fig. 3. SDS-PAGE analysis of native and modified *Par j* I. Lane A) native *Par j* I (0.5 mg/ml); lane B) modified *Par j* I (0.4 mg/ml); lane C) *P. judaica* extract (1 mg/ml).

tion (alum) for slow allergen release from the injection site was one of the first attempts (29). Allergens polymerized by reaction with aldehydes were subsequently developed, offering reduced allergenicity with retained immunogenicity. However, these derivatives always had high molecular masses (11, 17, 19).

With improvements in pharmaceutical technology, new formulations (tablets, capsules, etc.) of allergenic extracts which do not have to be injected and can be self-administered by patients using alternative routes (nasal, buccal including sublingual) are now widely used (2, 27). It is likely that the therapeutic action of these products is linked to the ability of allergenic components to pass through the mucosal barrier. The high molecular mass of polymerized allergens could hinder this passage, but native allergens involve a higher risk of side-effects because they reach the systemic circulation fast (21, 22). Therefore, in any strategy aimed at obtaining allergens for administration by routes other than parenteral injection, an important goal would be to

develop derivatives with lower allergenicity but fully preserved immunogenicity, and the same molecular size as the native form.

Chemical modification of allergens with KCNO, under conditions that promote the selective substitution of the ϵ -amino groups of the lysine residues on the surface of the protein molecules, appears to be an ideal way of developing a product meeting these requirements. In this process, the reaction time is important. There appears to be a close correlation between the reaction time and the degree of ϵ -amino group substitution, the optimal time being 16 h (unpublished data). In these conditions, the degree of $-\text{NH}_2$ group substitution, determined by the TNBS procedure, is more than 80%. This significantly reduces the allergenic potential of the modified allergens, whether they are in a mixture (extract) or well defined, such as OA and *Par j* I. It is also likely that $-\text{NH}_2$ group substitution makes the modified protein more stable, as a result of higher resistance to enzymatic breakdown, ϵ -amino groups being the preferential site of attack of some proteolytic

enzymes (20). Certain allergens, for example, *Der p* I, one of the major allergens of *D. pteronyssinus*, are particularly susceptible to rapid proteolytic degradation (25). Our *in vitro* (RAST inhibition) and *in vivo* (PCA) experiments clearly showed the diminished IgE-binding activity of the carbamylated allergens.

A possible cause of the diminished allergenicity of carbamylated allergen may be the loss of ionic charge of the ϵ -lysyl residues, which are converted into uncharged, even if highly polar, ureido groups. It has been reported that many epitopes contain, in their amino acid sequence, highly charged and polar groups. Furthermore, considering the influence of ϵ -amino groups of the lysine residues on the hydrophilic character of a molecule, and in view of its importance in the expression of an antigenic determinant (12, 13), it is also possible that carbamylation, acting on these groups, alters the hydrophilicity, consequently making the modified allergen less IgE reactive.

We cannot yet explain the remarkable difference in the degree of inhibition induced by carbamylated allergens in the two experimental models (RAST inhibition and PCA) used. While it is relatively easy to interfere at the level of RAST inhibition (modified allergen is from 211 to 1933 times less active), the carbamylated allergens undoubtedly have less effect on the PCA system (12.8 and 14.1), though there was a significant difference between native and modified forms. This is not really surprising because it is a common practical finding that any *in vitro* response is more susceptible to inhibition than an *in vivo* one.

Another observation arises from the PCA titer and C_{50} values. These parameters are the result of different interactions: in the RAST model, the affinity patterns of IgE antibodies present in human serum are directly involved, while in the PCA system one measures the effect of mediator release, resulting, in turn, from an interaction between mouse IgE and specific receptors on mast cells. A third possibility is that human IgE and mouse IgE recognize different epitopes on the allergenic molecule, and the KCNO reaction may have modified only some of these. Nevertheless, modified allergens appear to maintain their full immunogenic properties, since IgG antibodies from rabbits immunized with these derivatives reacted with the same components, including *Par j* I, recognized by IgG from rabbits immunized with native allergen, in immunoblotting experiments.

These features are by no means a prerogative of carbamylated allergens. Other procedures are available to achieve a worthwhile reduction of the IgE-binding properties of allergenic preparations while preserving their immunogenic capacity.

Formaldehyde was one of the first reagents, and glutaraldehyde was also used to modify single allergens and mixtures. The resulting derivatives had low residual allergenicity with retained immunogenicity, but their molecular masses tended to be very high because of extensive cross-linking between the protein components. This method of protein modification involves a very complex chemical reaction, as demonstrated by the instability and the high degree of polymerization of the derivatives, that consequently makes difficult their physico-chemical characterization. On the contrary, the KCNO method, being based on a defined chemical reaction with a specific target (ϵ -amino group of the lysine), has the remarkable advantage of giving rise to more easily standardizable modified products.

In fact, we have to observe that the more important aspect distinguishing the KCNO-modified allergen is the preservation of its native monomeric nature, as confirmed by SDS-PAGE experiments with OA and *Par j* I allergens. In these experiments, the choice of single allergens, rather than an allergenic extract, was justified by the fact that these allergens are available in a fairly pure form, so any change in molecular size induced by the KCNO reaction should be more clearly detectable. It would have been harder to note differences in molecular mass using an allergenic mixture, which contains several protein components of different dimensions.

The preservation of the native monomeric nature, combined with the lower allergenic potency and probably greater resistance to enzymatic degradation, is very important, making the KCNO-modified allergens particularly suitable for use by the recently introduced transmucosal routes, and not requiring injection.

In clinical pharmacology, the sublingual route is widely used; it is an effective method of giving drugs: the drug is absorbed, avoiding hepatic first-pass metabolism. The allergen has been found in the systemic circulation after nasal or sublingual administration to experimental animals (21, 22). Absorption of a radiolabeled allergen after sublingual administration follows a kinetic pattern, showing rapid appearance of the allergen in the systemic circulation which persists for several hours (22). This is probably what gives rise to the side-effects when native allergen is administered to allergic patients by this route. Theoretically at least, this should not happen with carbamylated allergens, in view of their lower allergenic activity.

This study provides good scientific support for the clinical use of KCNO-modified allergens in local hyposensitization trials in order to verify their real effectiveness.

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