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Physicochemical and immunologic characterization of low-molecular-weight allergoids of *Dactylis glomerata* pollen proteins

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Background: Orchard grass (*Dactylis glomerata*) pollen proteins were chemically modified by means of acid anhydrides (maleic and succinic anhydride) to obtain low-molecular-weight allergoids. Chemical modification in both cases led to the replacement of one positive charge (ϵ amino group of Lys) by one negative charge, yielding proteins with changed physicochemical properties in comparison to the native orchard grass-pollen proteins.

Methods: Physicochemical characterization of derivatives was done by gel chromatography, SDS-PAGE, and isoelectric focusing. To examine the IgE-binding properties of these derivatives, we carried out immunoblotting. To examine the ability of derivatives to induce IgG production, we immunized rabbits. Skin prick testing with the allergoids was performed on 15 individuals allergic to orchard grass pollens and on two healthy subjects.

Results: It was shown that the modified proteins retain their original molecular weights, but change pI to more acidic values. In the case of allergoids, a strong reduction in IgE binding was found. Immunization of rabbits with allergoids showed that the derivatives retain the ability to induce IgG production, and that the antisera obtained in such a way react to native (unmodified) extract. The ability of derivatives to induce allergic reaction was significantly reduced. The patients (86.6%) included in our study exhibited less than 50% of native extract response. Among them, 53.3% had no response to one or both allergoids.

Conclusions: These modification procedures yield allergoids with a reduced allergenic activity and preserved immunogenic potential suitable for use in immunotherapy.

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Conventional immunotherapy with subcutaneously injected allergens has long been used in treating allergies. One of the main problems in allergen-specific immunotherapy is the appearance of a local and systemic anaphylactic reaction even with very low doses (1). For a group of highly sensitive patients, it is even impossible to achieve a high dosage of allergenic extracts. Therefore, chemically modified allergens (allergoids) have been introduced in immunotherapy. The basic idea is that allergoids should display a considerably reduced allergenicity (specific IgE binding), yet retain the ability to induce the production of allergen-specific "blocking antibodies" of the IgG class (immunogenicity) (2–4).

Previous studies in immunotherapy with allergen extracts have shown that the production of IgG (mainly subclasses IgG₁ and IgG₄), besides some other factors, may be responsible for the relief of allergic symptoms (5–7). Several studies were performed to confirm the safety and efficacy of immunotherapy carried out with HMW (high-molecular-weight) allergoids prepared by means of aldehydes. It has been shown that allergoids are much better tolerated than the native allergen extract: fewer injections are required to reach higher dosages and allergenic activity is greatly reduced (8–14). A 2-year, double-blind, placebo-controlled study of immunotherapy with an alum-adsorbed *Parietaria* pollen allergoid (prepared with formaldehyde) showed that the allergoid does not appear to stimulate allergen-specific IgE production, as has often been reported for an unmodified allergen preparation (15).

In recent years, several reports have dealt with monomeric chemically modified allergens obtained by means of potassium cyanate (KCNO) (16). Relatively low molecular weights (monomeric allergoids) make them able to cross biologic membranes, although in small percentages in comparison to i.v. injected allergoids (17). Due to their oligomerized nature, such preparations may be suitable for sublingual (bucal) administration, as an alternative approach to HMW allergoids in treating allergic diseases.

The chemical aspects of the syntheses of various acid anhydride derivatives of *Phleum pratense* (timothy grass)-pollen extract proteins have been thoroughly described, and they have been proved not to cause oligomerization of proteins (18). We have continued to work with *Dactylis glomerata* (orchard grass) pollen, which is another very important cause of allergic diseases in Yugoslavia (19).

In this study, we have characterized (physicochemically and immunologically) the low-molecular-weight (LMW) allergoids prepared from orchard grass-pollen allergens by means of succinic anhydride and maleic anhydride. Intro-

duction of a negative instead of a positive charge seems also to cause changes in allergenic proteins resulting in allergoids with different properties in comparison to a native extract.

Material and methods

Preparation of extract

Pollen samples obtained from the Institute for Immunology and Virology, Torlak, Belgrade, Yugoslavia, were collected from orchard grass in the Belgrade area. Dry (defatted by ethyl ether) pollen (1 g) was suspended in 10 ml distilled water and gently shaken for 3 h at 18°C. After spinning, the sample (Dg) was stored at –20°C until use.

For immunization of rabbits, the extract was always freshly prepared in distilled water.

Preparation of allergoids

For allergoid preparation, 1 mmol of chemical agent (maleic and succinic anhydride, yielding M-Dg and S-Dg allergoid) was used for each 2 mg of protein. The reaction was carried out for 1 h; appropriate anhydrides were added in six portions, and gently shaken at 4°C. The pH value of the reaction mixture was adjusted after each portion to pH 8 with 10 mM NaOH. All modified extracts were dialyzed against PBS, pH 7.4, for 24 h and then spun. The protein concentration was determined by Bradford's method (20) and then adjusted to 1 mg/ml for all modified extracts.

All samples were stored at –20°C until use.

Molecular weight distribution

The molecular weight distributions of native extract (Dg) and allergoid preparations (M-Dg and S-Dg, respectively) were compared by analytic chromatography on Sephadex G-150 SF (Pharmacia, Uppsala, Sweden). Desalting of the samples was carried out by analytic chromatography on Sephadex G-25 (Pharmacia, Uppsala, Sweden). Only fractions with positive Bradford assay were pooled and applied on the Sephadex G-150 column. The column (2 × 70 cm) was equilibrated with 0.9% NaCl at room temperature. The flow of 12 ml/h was maintained during chromatography, and 4-ml fractions were collected with an automatic collector under continuous UV monitoring at 280 nm.

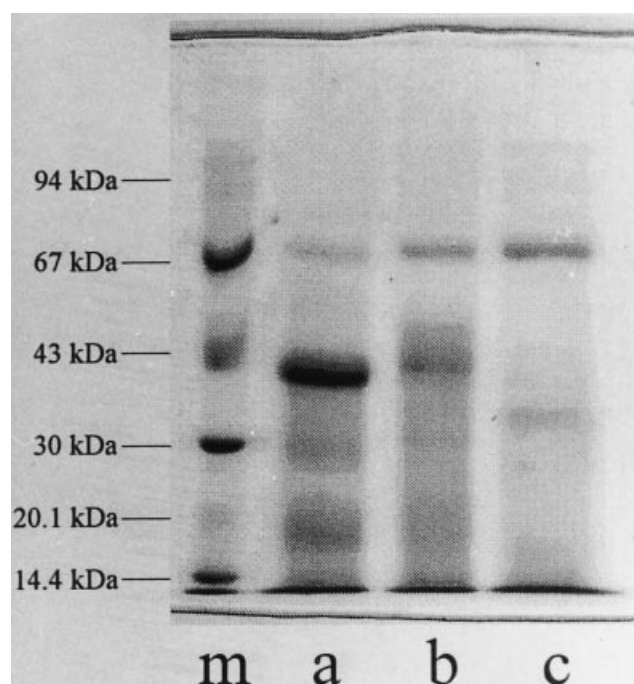


Figure 1. SDS-PAGE electrophoretic analysis of native and modified extracts. Lane a) native Dg extract; b) modified M-Dg; c) modified S-Dg; m) molecular weight markers.

Antisera production

The antibodies against whole extract of orchard grass pollen and allergoids were raised in rabbits according to Harboe & Ingild (21). The animals were injected with 0.5 ml of a 1:1 emulsion of pollen extract (1 mg/ml) in complete Freund's adjuvant. The injections were repeated after 14, 28, and 42 days and every 2 weeks with an incomplete adjuvant. Bleeding was performed 50 days after the first immunization and every 2 weeks thereafter. The presence of antibodies to orchard grass-allergen proteins was determined by immuno-

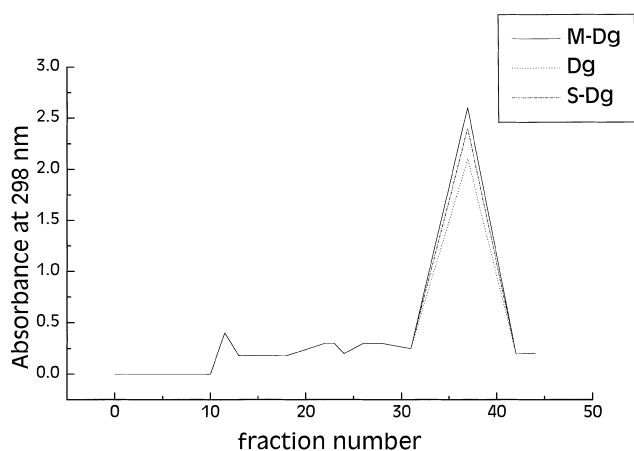


Figure 2. Molecular weight distribution on Sephadex G-150 of allergoids and allergen preparations.

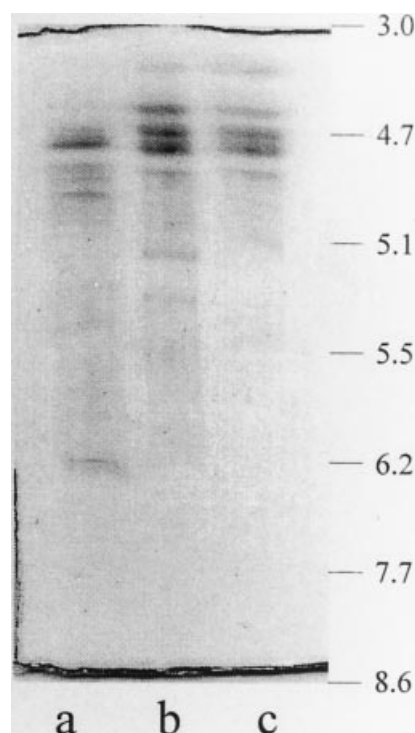


Figure 3. IEF analysis of native and modified extracts. Lane a) native Dg; b) modified M-Dg; c) modified S-Dg.

diffusion. The sera obtained from two rabbits immunized with the same antigen mixtures were mixed in equal volumes and partially purified by ammonium salt fractionation.

Rocket immunoelectrophoresis

About 10 μ l of samples was applied to 1% agarose gel containing 17 μ l of rabbit antibodies per cm^2 , on a 5 $\times$ 7 cm glass plate. Electrophoresis was carried out in a buffer containing 5 mM barbital, 25 mM Na-barbital, pH 8.6, for 16 h at 2 V/cm.



Figure 4. Rocket immunoelectrophoresis of native and modified extracts. A) rabbit anti-Dg serum; B) rabbit anti-M-Dg serum; C) rabbit anti-S-Dg serum: a) native Dg, b) M-Dg, c) S-Dg.

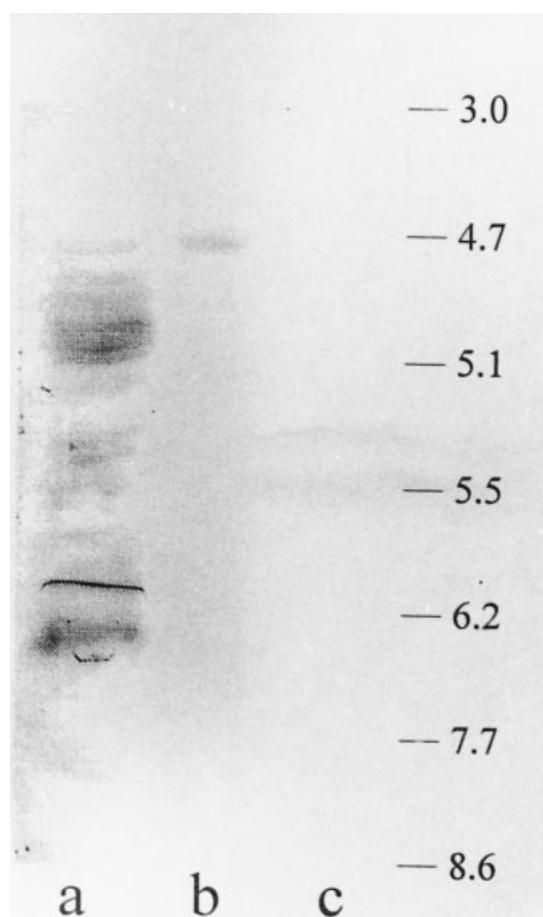


Figure 5. IgE-binding components from IEF-separated proteins of native and modified extracts. Lane a) native extract; b) M-Dg; c) S-Dg.

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

SDS-PAGE was performed in a vertical slab gel 4/10%, according to Laemmli (22), in a Hoefer scientific instrumentation apparatus for a modified and unmodified extract with the same sample volume. SDS-PAGE was performed under reducing conditions at 80 V for 1 h and 250 V for another 2 h. The gel was stained with 0.05% Coomassie brilliant blue R-250 in water:methanol:acetic acid (50:40:10).

Isoelectric focusing (IEF) and printing to nitrocellulose

The IEF of samples was performed in 5% polyacrylamide slab gel (1 mm) in the presence of 2% Amfolites (Pharmacia, Uppsala, Sweden), pH 3.5–10, in a Multiphor electrophoretic system at 2000 V for 4 h. Each sample was applied in quantities of 20 g per well. The pH gradient was determined by cutting the gel into strips and incubating it in distilled water for 30 min. The proteins were subsequently trans-

Table 1. Evaluation of *in vivo* allergenicity of native and modified extract by SPT

Grading system for puncture skin testing using histamine as reference	
0	No discernible wheal
1+	<1/2 histamine diameter
2+	1/2 histamine and <histamine diameter
3+	size of histamine wheal $\pm$ 1 mm
4+	>histamine diameter and <2 $\times$ diameter
5+	>2 $\times$ histamine control

ferred to nitrocellulose membrane (Serva, Germany) by capillary force overnight. Blots were blocked in TBS-0.1% Tween 20 for 1 h and dried until development.

Human sera

Sera from 15 patients (nine male and six female), with documented clinical histories of orchard grass-pollen allergies, included in skin prick tests (SPT) with allergoids, were pooled for immunoblot experiments.

Immunoblotting and IgE binding

IgE-binding bands were determined by probing with 10-fold diluted pool human sera (pool of the sera of 15 patients included in SPT) in diluting buffer (PBS-0.5% BSA). As secondary antibodies, we used HRP-labeled anti-IgE (Sigma Chemical Co., St Louis, MO, USA). The binding pattern was visualized by a substrate solution consisting of 0.06% w/v diaminobenzidine and 0.03% hydrogen peroxide in 50 mM Tris, pH 7.6.

Enzyme determination

Alkaline phosphatase activity was assayed in native and modified extracts with 3 mM *p*-nitrophenyl phosphate in 50 mM Tris buffer and 5 mM MgCl₂ at pH 10.3. Enzyme activity was measured by mixing an initial 20 μ l of the sample with 100 μ l of the appropriate enzyme substrate. Absorbance at 405 nm was measured after 60-min incubation at 25°C.

SPT

Fifteen patients allergic to grass pollens and two healthy subjects were included in the study. SPT were performed on the volar surface of the forearm with allergoids and

Table 2. Evaluation of *in vivo* allergenicity of native and modified extract by SPT

Sample	Patients														
	Group 1								Group 2					Group 3	
	3+	3+	3+	2+	2+	2+	2+	3+	4+	4+	4+	5+	5+	3+	2+
Dg	3+	3+	3+	2+	2+	2+	2+	3+	4+	4+	4+	5+	5+	3+	2+
M-Dg	2+	0	1+	0	0	0	0	0	2+	2+	2+	3+	3+	2+	1+
S-Dg	0	2+	0	0	0	2+	0	0	2+	2+	1+	2+	3+	2+	1+

unmodified extract of orchard grass pollen. The samples were prepared in the same manner in PBS and glycerol, yielding a final protein concentration of 0.33 mg/ml. Histamine (10 mg/ml) was used as positive control and saline (0.9%) as negative control. The results of SPT were read after 20 min. Wheal diameters were expressed relative to the histamine response [23].

Results

Molecular weight distribution

In SDS-PAGE stained with CBB R-250 (Fig. 1), samples S-DG and M-DG showed complex and mutually similar patterns different from sample DG (control). S-DG and M-DG showed the shift of some major protein bands toward slightly higher molecular weights (about 1–2 kDa). S-Dg sample lacked one major protein band (about 34 kDa). The molecular weight distributions determined by gel chromatography expressed no significant oligomerization or cross-linking of proteins caused by the modification procedure. Protein profiles in gel chromatography were the same for native and modified extracts (Fig. 2).

IEF

All samples on IEF gel stained with CBB R-250 (Fig. 3) showed a similar shift in pI values, in comparison with the control sample, toward more acidic values. M-DG and S-DG showed some acidic protein bands (pI values of 3.3–5.4 for S-Dg and 3.4–5.9 for M-Dg). S-DG and M-DG patterns were mostly similar but differed from control. There were no protein bands in the basic region in allergoid preparation. In the control sample, the most acidic band was at a pI of about 3.8.

Rabbit IgG binding

Rocket immunoelectrophoresis in a gel containing antibodies prepared to M-Dg and S-Dg is shown in Fig. 4B and C,

respectively. Modified samples cross-reacted, and the antibodies prepared in such a way cross-reacted with native extract, recognizing both its acidic and basic proteins. The number and height of the rockets were similar in a preparation of both allergoid preparations. Rabbit antisera raised against native extract also reacted to modified samples, but the number of precipitation lines was reduced in the case of both allergoids (Fig. 4A). Rabbit antisera raised against native extract produced two precipitation lines with the S-Dg sample (Fig. 4A, c) and four with the M-Dg sample (Fig. 4A, b).

Human IgE binding of allergens and allergoids

The bands printed to nitrocellulose, after IEF, binding IgE in native extract are shown in Fig. 5. The bands in the S-Dg and M-Dg samples were barely visible. Only one band was visible in the M-Dg sample at a pI of about 4.7.

Chemical modification with acid anhydrides led to a considerable reduction of allergenic potency in 15 allergic patients included in our study.

Enzyme determination

Alkaline phosphatase activity in allergoids was less than 50% of that in native extract. In the S-Dg sample, phosphatase activity was 46% of the activity in native extract, while in the M-Dg sample it was only 33%.

In vivo allergenicity of native and modified allergenic extracts

Two healthy patients included in the study showed no response to the allergoids and allergen preparations. There were no toxic or other side-effects during testing and 6 months thereafter.

Our patients can be divided into three response categories. Eight patients (53.3%) failed to respond to one or both allergoids, another five patients (33.3%) had less than 50% of response to native extract, and two patients (13.3%) responded to native extract within a range of 50–75% (Table 2).

Discussion

The immunogenic properties of allergoids are similar to those of native allergenic extracts but have a considerably diminished allergenic potency, making them safer in the course of specific immunotherapy. Side-effects are considerably reduced, and it is possible to achieve a high maintenance dosage of the (modified) extract. Only HMW allergoids (obtained by oligomerization of allergen molecules by means of glutaraldehyde and formaldehyde) have been used so far in immunotherapy.

Recently investigated alternative ways of administering allergens during specific immunotherapy (sublingual) excluded HMW allergoids. Little is known about the pattern of absorption after the sublingual administration of allergen, but oligomerized allergoids presumably may have serious problems in passing through mucous membrane. More recent reports deal with new allergoids prepared by means of KCNO-producing allergoids with LMW allergoids, and can be used in alternative treatment of allergies.

In our study, we have thoroughly characterized a new type of allergoids prepared by means of acid anhydrides which does not cause aggregation or cross-linking of proteins.

We have investigated, *in vitro* and *in vivo*, physicochemical, antigenic, and allergenic properties of two different preparations of orchard grass-pollen allergoids. Both types of allergoids were obtained by mild treatment with the same quantities (1 mmol/mg proteins) of succinic anhydride and maleic anhydride and the same length of exposure (1 h) to the modifying agents.

The physicochemical characterization of the derivatives has been arrived at by SDS-PAGE and IEF. As expected, the derivatives showed an acidic shift in IEF (Fig. 3). In SDS-PAGE patterns (Fig. 1), S-Dg and M-Dg protein bands exhibited apparently higher molecular weights. This phenomenon is present in the case of very acidic proteins, because the introduction of many negative charges leads to incomplete SDS binding and reduced electrophoretic mobilities (24). This was confirmed by the results of gel chromatography on Sephadex G-150 (Fig. 2), in which we have demonstrated that the proteins retain their original molecular weights; i.e., no noncovalent oligomerization of proteins occurs.

Immunization of rabbits shows that allergoids may induce IgG response, and that antibodies prepared in such a way recognize native extract; i.e., the modified proteins retain all their immunogenic properties by retaining some of the antigenic determinants present in the native extracts. The rabbits' response to allergoids was comparable to native

extract response in number and height of the rockets (Fig. 4). Moreover, antisera raised against one allergoid recognized another, suggesting relatively similar antigenic epitopes in both types of allergoids. Apparently, the very different response of the rabbits to allergoids (Fig. 4) in comparison to allergen extracts was based on the changed physicochemical features of allergoids. Modified proteins exhibit a very narrow range of pI values (Fig. 2), which cannot be distinguished by the rocket immunoelectrophoresis used in this study.

The human IgE binding of allergoids, evaluated by immunoblot after IEF, showed no band capable of binding IgE from allergic patients' sera in the case of S-Dg allergoid (Fig. 5), and one such band in the case of M-Dg.

The SPT response to unmodified extract varied from 3+ to 5+ and in the case of allergoids from 0 to 3+ (Table 2). The strongest response by all patients was to native extract. The SPT results indicated that S-Dg and M-Dg have similar abilities to provoke (or not to provoke) allergic reaction. Skin reaction to the allergoids increased with dosage (results not shown), but it was slower than the reaction to the native extract. In view of the possible therapeutic usefulness of these allergoids, the very important fact is that, in spite of the existing diversity of patient responses, there was no patient with stronger response to the allergoid preparations than to the native extract. The difference in the degree of reduction of IgE binding, as seen in the two experimental models (immunoblot and SPT), was not unexpected, because, in practice, the *in vitro* response is more sensitive to the reduction of IgE binding than the *in vivo* one. This is in agreement with the results obtained by other authors for modified allergens in RAST inhibition and PCA (17, 25). One of the reasons may be the total complexity of the *in vivo* response to allergen challenge.

A possible explanation of the reduction of allergenicity in the case of *in vitro* and *in vivo* tests may be the conversion of the positively charged amino group of Lys into a more voluminous, negatively charged group. It has been reported that many epitopes contain highly charged or polar groups (26). In the case of carbamylation, the conversion of the Lys amino group into a polar but uncharged ureido group can diminish allergenicity. It may be added that the inversion of the epitope charge can also alter IgE binding, probably with the influence of steric hindrance (the new group is significantly more voluminous than the lysyl group). These effects influence the conformation of the enzyme active site as well, and lead to a reduced activity in the allergoid preparations (demonstrated in the case of alkaline phosphatase).

Finally, it can be concluded that maleic anhydride and succinic anhydride modify proteins without causing oligomerization and related precipitation. Our study confirms

that this type of modification procedure yields allergoids of good performance in immunotherapy. Experiments in rabbits show the retained ability of the allergoid preparations to provoke the response of IgG antibodies. Regarding possible immunotherapy, where the induction of "blocking IgG antibodies" can be anticipated, this experimental finding is very important. Our experiments of IgG binding (not mentioned here) from the sera of the patients who are undergoing specific immunotherapy to grass pollens (patients on the maintenance dosages) show that the ability of the allergoid preparations to bind human IgG antibodies is comparable to the native extract (qualitatively and quanti-

tatively), thus leading us to believe that IgG epitopes in allergoids are mostly retained for successful immunotherapy (these results are in excellent agreement with our experiments performed in rabbits [Fig. 4A]).

Therefore, since some patients distinguish two types of allergoids, physicochemically very similar, and may have a strong response to only one of them and no response to the other, it is desirable to test patients before immunotherapy in order to choose the right allergoid. A transmucosal passage experiment should be done for this type of allergoid. Further reduction of the allergenic potential of allergoids prepared in this way is under investigation.

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