

Original article

The influence of a residual group in low-molecular-weight allergoids of *Artemisia vulgaris* pollen on their allergenicity, IgE- and IgG-binding properties

Background: Reaction of ε -amino groups of lysine with potassium cyanate, maleic, or succinic anhydride leads to allergoids of low molecular weight. No study has been performed to compare their properties and investigate the influence of a residual group on allergenicity and human IgE- and IgG-binding of these derivatives.

Methods: Allergoids of a pollen extract of *Artemisia vulgaris* were obtained by means of potassium cyanate, and succinic and maleic anhydride. Biochemical properties were investigated by determination of amino groups, enzyme activity, isoelectric focusing IEF and SDS-PAGE. IgE- and IgG-binding was determined using immunoblots and ELISA inhibition. Allergenicity was investigated by skin prick tests (SPT) on a group of 52 patients, of which 6 were control subjects, 30 were patients with no previous immunotherapy (IT), and 16 were patients undergoing immunotherapy.

Results: The same degree of amino-group modification (more than 85%), residual enzyme activity (less than 15%), IEF, and SDS-PAGE pattern were noted. In the immunoblots of IgE-binding, there was more pronounced reduction in the succinyl and maleyl derivatives than in the carbamyl one. IgG-binding was less affected by carbamylation than by acid anhydride modification. The SPT showed that the succinylated derivative had the most reduced allergenicity (98% showed a reduced wheal diameter when tested with the succinyl derivative, 87% with the maleyl allergoid, and 83% with the carbamyl allergoid). The most significant difference among allergoids could be seen in the group of patients with high skin reactivity (83% of patients showed no reaction to the succinyl derivative when compared to the value of 28% for the carbamyl derivative or 22% for the maleyl derivative).

Conclusions: According to our results, all three modification procedures yielded allergoids with a similar extent of modification. No single biochemical parameter investigated in the study could predict the degree of reduced allergenicity *in vivo*. The most reduced allergenicity was seen in the succinyl derivative while the preservation of IgG binding epitopes was of the highest degree for the carbamyl derivative.

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Key words: allergoid; *Artemisia vulgaris*; chemical modification; maleic anhydride; mugwort pollen; potassium cyanate; skin prick testing; succinic anhydride.

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The concept of chemically modified allergens (allergoids) was introduced into clinical practice several decades ago in order to reduce possible side effects of conventional subcutaneous immunotherapy (IT) (1, 2). The first clinically introduced allergoids were obtained by formaldehyde treatment and were known to be of higher molecular weight than native proteins due to cross-linking and polymerization caused by a modification reaction with aldehydes. Their clinical efficacy and safety were documented in several controlled clinical trials and they were safe and reliable in IT of allergic disorders (3–10).

Recently, allergens have been administered in non-injectionable forms (for different types of local IT) in

routine practice, especially because they have good tolerance and safety, and proved benefits for patients (11–14). Although tolerated well by allergic patients, several side effects have been reported, mainly relating to the gastrointestinal tract and mouth (11, 15).

For these reasons, various studies have been performed to develop chemically modified allergens suitable for use in different types of local IT. The main properties of such derivatives should be, as well as reduced allergenicity and retained ability to induce an IgG response (immunogenicity), the retention of native molecular weight (in order to pass mucosal barriers). The derivatives that fulfil these criteria are known as low-molecular-weight allergoids

and are obtained by carbamylation (16, 17) or acid anhydride treatment (18).

A study concerning the pharmacokinetics of a monomeric carbamylated allergoid derived from *Parietaria judaica* showed similar behavior of the allergoid and the allergen concerning their local kinetics, whereas the plasma peak was higher for the allergoid than for the allergen (19). Clinical trials of local (carbamylated) allergoid IT proved its efficacy in mite-induced rhinoconjunctivitis (20) and the treatment of asthmatic symptoms in allergic children (21). A satisfactory safety profile of sublingual IT with a monomeric allergoid in adults has been confirmed in a multicenter postmarketing surveillance study (22).

Maleylated and succinylated low-molecular-weight allergoids of *Dactylis glomerata* showed marked reduction of IgE-binding when compared to native allergens as demonstrated *in vivo* by skin prick test (SPT) (18).

This study compares the biochemical and immunological properties of three different types of low-molecular-weight allergoids prepared from *Artemisia vulgaris* Av pollen proteins. The main goal was to evaluate their allergenicity *in vivo* as well as IgE- and IgG-binding properties *in vitro*, to investigate whether the acid anhydride derivatives have comparable properties to the more evaluated carbamylated derivatives, which could make them possible candidates in future IT.

Material and methods

Extract preparation

Pollen samples obtained from the Institute for Immunology and Virology, Torlak, Belgrade, Yugoslavia, were collected from mugwort weed in the Belgrade surroundings. Pollen (5 g) was defatted by ethyl ether, suspended in 50 ml of distilled water and shaken at 4°C for 6 h. The suspension was centrifuged at $10\,000 \times g$ for 30 min. The supernatant was stored at -20°C until required.

Modification procedures

A mugwort pollen extract (10 mg) was modified by Potassium cyanate (KCNO) treatment according to a previously published procedure (16). The modified sample designated KCNO-Av was dialyzed against water and concentrated to the protein concentration of 1 mg/ml.

The same protein quantities were used in modification with 400 mM succinic and maleic anhydrides according to a previously described procedure (18). The final samples, designated Mal-Av and Succ-Av were dialyzed against water and adjusted to the protein concentration of 1 mg/ml.

Protein and enzyme determinations

Protein concentrations were determined by the Bradford method (23). Enzyme activities were determined in freshly prepared samples before freezing.

Acid phosphatase activity was determined with 1 mg/ml nitrophenyl phosphate (4-NPP) as the substrate in 50 mM sodium

acetate (NaOAc) buffer, pH 5.2. The reaction was stopped after 10 min with the addition of 3 M sodium hydroxide and absorbance was read at 405 nm.

Determination of amino groups

The number of amino groups in control and modified samples was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS) as the reagent according to a previously described procedure (24). For determination of amino group, the protein concentration in modified and unmodified samples was adjusted to 0.1 mg/ml. Results were expressed as means of three different determinations and for modified samples as a percentage of the number of amino groups determined for the unmodified sample (expressed as 100%).

Isoelectric focusing

Isoelectric focusing (IEF) was performed in 5% polyacrylamide slab gel (1 mm) in the presence of 2% Amfolites pH 3.5–10 (Pharmacia, Uppsala, Sweden), in a Multiphor II electrophoretic system at 2000 V for 4 h with a standard protein mixture (Pharmacia, Uppsala, Sweden) for determination of the pH gradient. Each sample was applied in a quantity of 10 µg per well. Gel was stained with Coomassie Brilliant Blue R-250 to visualize the separated proteins.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotting to nitrocellulose membrane

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (25) using a Hoefer scientific instrumentation apparatus with a discontinuous buffer system. Native and modified samples in the same quantities of 10 µg per well (Av, KCNO-Av, Succ-Av and Mal-Av) were diluted in sample buffer (5% β-ME) and boiled for 5 min before the run. Protein components were resolved on a 10% gel with a standard protein mixture for mol. wt. reference at 80 V for 1 h and 250 V for another 2 h. Gels were stained with Coomassie Brilliant Blue R-250 to visualize the separated proteins.

Resolved components were blotted by a semidry electroblotter on the nitrocellulose paper. Transferred proteins were blocked in TBS-0.1% Tween 20 for 1 h and dried until development.

Study population

Fifty-two subjects were included in the study (24 men and 28 women; aged 15–65). The SPT involved: 6 allergic volunteers with negative SPT to mugwort pollen extract (as a control group); 30 patients with no previous IT received (group 1); and 16 patients in the course of IT to mugwort pollen (12 on sublingual/oral IT). The 46 patients included in the study were suffering from mugwort pollen allergy. The patients were mainly poly sensitized (only 4 showed monosensitization to mugwort pollen) to common aeroallergens and were also SPT positive to various fruits and vegetables (data not shown). They showed a history of the following allergic diseases (46 patients, control group excluded): allergic rhinitis (27), allergic asthma (6), allergic urticaria (2), allergic rhinoconjunctivitis (9) and allergic conjunctivitis (2), in accordance to the Position Paper for the revised nomenclature for allergy (26).

Skin prick testing

The samples for SPT were prepared at the Institute for Immunology and Virology, Torlak, Belgrade as described previously (18). Wheal

diameters were expressed in relation to the histamine response (27) using the same grading system as described. SPT were performed during the period from July to November.

Human sera

Of the 46 people in the SPT study, the sera from 30 patients allergic to mugwort pollen with a documented clinical history of mugwort pollen allergy and with no previous recorded IT to mugwort pollen were pooled for immunoblot experiments (pool I). Sera from another 16 patients allergic to mugwort with documented clinical history of mugwort pollen allergy and in the course of specific, subcutaneous, or oral IT were pooled for immunoblot experiments (pool II). The values for total and mugwort pollen-specific IgE as well as eosinophilic cationic protein (ECP) in pooled sera I and II were determined by the UniCap (Pharmacia, Uppsala, Sweden) system.

Sera from 10 patients (CAP class 4, 5 or 6 to mugwort pollen) were selected from the group of SPT tested patients. Patients were allergic to mugwort pollen, with documented clinical histories of mugwort pollen allergies (allergic rhinitis, allergic asthma, allergic urticaria, allergic rhinoconjunctivitis, and allergic conjunctivitis). The patients' sera were pooled and used in the ELISA and ELISA inhibition experiments (pool III).

IgE and IgG detection

IgE and IgG binding bands were probed in immunoblot. The proteins were resolved by SDS-PAGE, transferred to nitrocellulose paper as described above and determined by probing with 10-fold diluted pooled human sera (pool I and pool II, separately) in a diluting buffer (TBS 0.5% BSA). As secondary antibodies we used alkaline phosphatase labeled monoclonal anti human IgE (Sigma Chemical Co, St Louis MO, USA) or alkaline phosphatase labeled anti-human IgG (Sigma Chemical Co, St Louis MO, USA). The binding patterns were visualized with a substrate solution consisting of 1.5 mg/ml bromochloroindolyl phosphate (BCIP) and 3 mg nitroblue tetrazolium (NBT) in a 100-mM Tris buffer, containing 150 mM NaCl, and 5 mM MgCl₂, pH 9.6.

ELISA and ELISA inhibition

ELISAs were performed as described previously (28). Briefly, the microtiter plates (Nunc, Maxi Sorp) were coated with 50 µl of mugwort extract (7 µg/ml) overnight at room temperature and washed with TBS 0.1% Tween 20. After blocking with 100 µl of 1% BSA in TBS 0.1% Tween 20, the 50 µl of five-fold diluted human sera pool III, incubated with 50 µl of serially five-fold diluted allergen (concentrations of 130 µg/ml to 0.0016 µg/ml) or allergoids (concentrations of 200 µg/ml to 0.0128 µg/ml) in tubes for 30 min at 4°C, were added to the plates. For ELISA inhibition experiments, human sera pool III was used in a dilution to achieve 80% of IgE-binding as determined in a separate saturation experiment. For inhibition, serial dilutions of unmodified and modified samples were prepared in a diluting buffer consisting of TBS 0.5% BSA. Human serum from subjects with negative skin tests to mugwort pollen extract was used as a negative control. Absorbance measurements were done at 405 nm after adjusting the background with a negative control. The inhibition percentage of inhibition was calculated as:

Percentage inhibition

$$= \frac{\text{OD of the test sample} - (\text{OD of the inhibited sample} / \text{OD of the test sample}) \times 100}{\text{OD of the test sample}}$$

The test sample (positive control) was 50 µl of five-fold diluted human serum incubated with 50 µl of a diluting buffer.

Antisera production

The antibodies against control (unmodified) and modified mugwort pollen extract proteins were raised in rabbits according to Harboe and Ingild (29). The presence of antibodies was determined by immunodiffusion.

Results

The number of amino groups and enzyme activities in allergoids

The biochemical properties of the allergoids obtained were compared for two different parameters: the extent of unmodified amino groups and the enzyme activity (acid phosphatases). Regarding the number of modified amino groups, all three derivatives showed the same extent of modification. The percent of modification was in the range of 85% for the succinylated and maleylated derivatives and 90% for the carbamylated derivative. The acid phosphatase activity was about 10% when compared to the unmodified sample in all three derivatives.

Isoelectric focusing and SDS-PAGE patterns

Isoelectric focusing of derivatives showed that the highest changes in the pI value when compared to unmodified pollen extract were obtained for the maleylated derivative (Fig. 1A). The pI ranges obtained for investigated derivatives were 3.5–5.2 (KCNO-Av), 3.5–5.0 (Succ-Av) and 3.5–4.8 (Mal-Av). Similar values were obtained for acid anhydride derivatives of *Dactylis glomerata* pollen proteins (18). Acid anhydride derivatives of mugwort pollen proteins showed the same shift in electrophoretic mobility in SDS PAGE (Fig. 1B) due to abnormal binding of SDS as demonstrated for *D. glomerata* allergoids.

ELISA inhibition

The IgE binding properties of allergoids *in vitro* were determined in an ELISA inhibition experiment with the mugwort pollen extract proteins coupled to the microtiter plate as the antigen. The extent of inhibition was expressed as the IC₅₀ value, i.e., the inhibitor concentration needed to obtain 50% of inhibition. The IC₅₀ values obtained for the unmodified extract, carbamylated, maleylated and succinylated derivatives were: 0.66 µg/ml, 4.3 µg/ml, 19.2 µg/ml and 45.4 µg/ml respectively. The relative inhibitory activities were as follows: unmodified proteins (100%), Succ-Av (54%), Mal-Av (68%), KCNO-Av (78%). The highest IC₅₀ value obtained for succinylated derivative means the lowest IgE binding potency (about 70 times lower than the value obtained for the unmodified extract) and suggests the lowest allergenicity (Fig. 2).

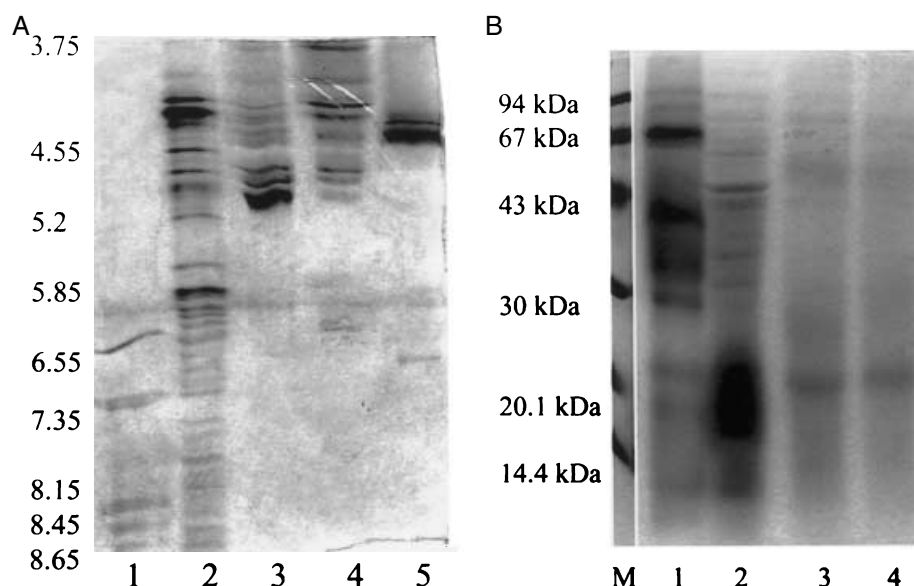


Figure 1. (A) Isoelectric focusing of native and modified samples. Line 1, pI markers; line 2, mugwort pollen extract; line 3, carbamylated proteins; line 4, succinylated proteins; line 5, maleylated proteins. (B) SDS-PAGE of native and modified samples. Line M, molecular weight markers; line 1, mugwort pollen extract; line 2, carbamylated proteins; line 3, succinylated proteins; line 4, maleylated proteins.

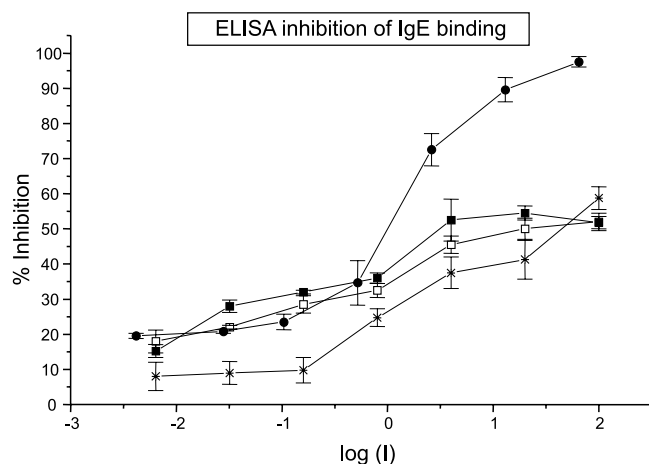


Figure 2. Determination of IgE-binding properties of native and modified samples by ELISA inhibition: mugwort pollen extract (●), carbamylated derivative (■), maleylated derivative (□), succinylated derivative (*).

Total and specific IgE and Eosinophilic cationic protein (ECP) values in pooled human sera

The values obtained for total IgE in pooled human sera I (no previous IT received) and II (patients on IT) were as follows: 329 kUA/l and 586 kUA/l. Specific IgE values to *Artemisia vulgaris* pollen were: 4.1 kUA/l (class 3) for pool I, and 12.8 kUA/l (class 3) for pool II. Higher values of total and specific IgE in pool II than in pool I were due to selection criteria for IT, because not all patients with allergic symptoms had started receiving IT.

The values for ECP were: 3.77 µg/l (pool I) and 2.23 µg/l (pool II). The blood samples used for determination of the ECP value were taken at the time of the SPT (from July to November). Despite the higher values of specific IgE in the group of treated patients, we demonstrated a lower value of ECP in the group, when compared to the values obtained for the untreated group of patients, which might suggest the success of IT.

IgE- and IgG-binding in immunoblot

The IgE-binding properties of allergoids *in vitro* were also determined in immunoblot. The proteins separated in SDS-PAGE were tested for IgE (Fig. 3A) and IgG binding (Fig. 3B) from two different pools of patient's sera: pool I (patients that never received IT) and pool II (patients in the course of IT to mugwort pollen). According to our results, IgE binding in all derivatives was significantly reduced when compared to the unmodified extract, especially in the succinylated and maleylated derivatives. There were some barely visible bands at about 60 kDa, supposedly for Art v 1, the major mugwort pollen allergen pI of 4.2 (30). Our opinion is that the lower content of amino groups in highly acidic proteins, such as Art 1, makes it less suitable for modification with agents acting on ε-amino groups of Lys (i.e. all modification agents which may result in a high degree of retention of native conformation). The same could be true for another allergen of mugwort pollen, Art v 2, which is also highly acidic protein. However, a problem with the dimeric protein Art v 2 is that it loses 95% of the IgE binding epitopes by

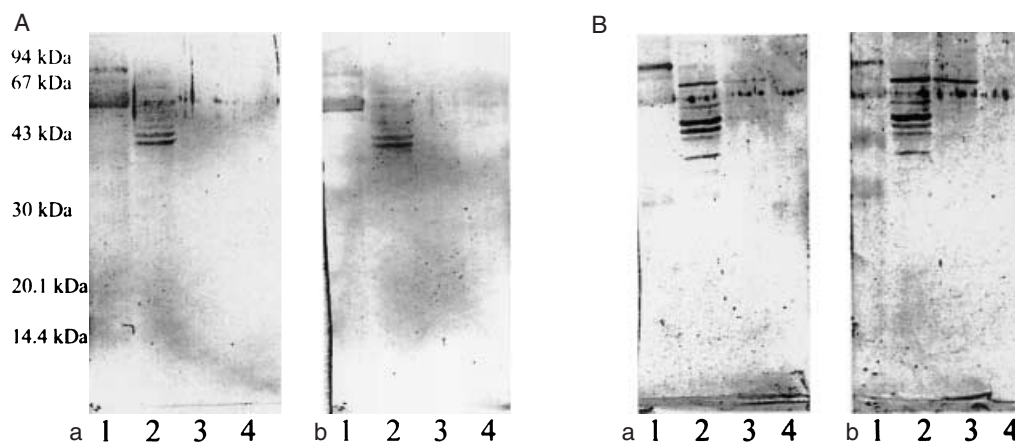


Figure 3. (A) Determination of IgE-binding properties of native extract and modified proteins from a pool of allergic patients (a) with no immunotherapy received and (b) in the course of immunotherapy. Line 1, mugwort pollen extract; line 2, carbamylated proteins; line 3, succinylated proteins; line 4, maleylated proteins. (B) Determination of IgG-binding properties of native extract and modified proteins from a pool of allergic patients with (a) no immunotherapy received and (b) in the course of immunotherapy. Line 1, mugwort pollen extract; line 2, carbamylated proteins; line 3, succinylated proteins; line 4, maleylated proteins.

denaturation (31, 32) and it is hard to believe that epitopes could behave differently in the modified protein.

IgG binding followed the same pattern in all investigated samples as for IgE binding. Mostly the same IgG binding bands could be seen in IgE binding experiments (Fig. 3B). There was no major difference in IgE and IgG binding between the different human sera pools tested in our study. Most of the IgE and IgG binding bands were nearly the same in the group of patients with no previous IT as the group of patients on the course of IT.

Determination of immunogenicity of allergoids

The mugwort pollen allergoids used for rabbit immunization were able to elicit an immune response. The antiserum obtained to unmodified allergen was able to recognize modified allergens in the experiment of double immunodiffusion. The antisera obtained to all three kinds of modified allergens also cross-reacted with unmodified proteins (Fig. 4). The results are in complete agreement with those of previous studies of immunogenicity of

succinyl and maleyl derivatives of *Dactylis glomerata* pollen proteins (18) as well as carbamyl derivatives of *Parietaria judaica* allergens (17).

Determination of allergenicity of allergoids

To determine the *in-vivo* properties of allergoids we performed SPT on 46 individuals allergic to mugwort pollen; the control group consisted of 6 allergic patients with negative SPT to mugwort pollen. There was no response to allergoids or the unmodified extract in the control group.

According to SPT results (Fig. 5) in the group of 46 patients 98% showed a reduced wheal diameter when tested to Succ-Av, 87% to Mal-Av, and 83% to KCNO-Av. A high percentage of tested patients showed no reaction at all to allergoids (82%, 50% and 63% respectively). There was a difference between the two groups of patients. The group of patients on the course of IT (group II in Fig. 5) reacted more intensely to allergoids (the difference was more pronounced for the carbamy-

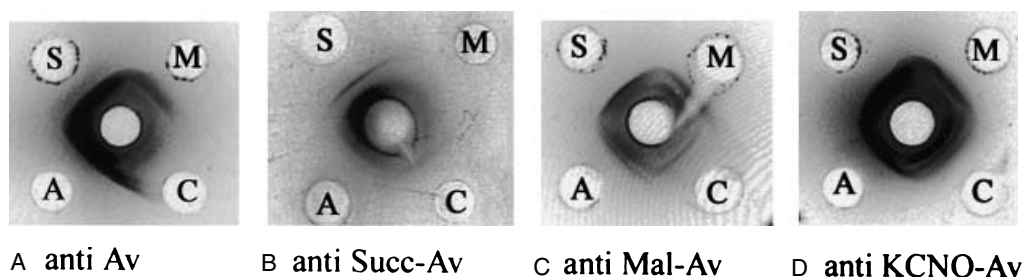


Figure 4. Determination of immunogenicity of allergoids by double immunodiffusion. The central wells contained 10 µl of raised antibodies designated as follows. Anti-Av: rabbit antisera to unmodified extract; anti-Mal: antisera to maleylated proteins; anti-Succ: antisera to succinylated proteins; anti-KCNO: antisera to carbamylated proteins. The four wells around the central well contained 10 µl of the following samples: A, mugwort pollen extract; C, carbamylated proteins; S, succinylated proteins; M, maleylated proteins.

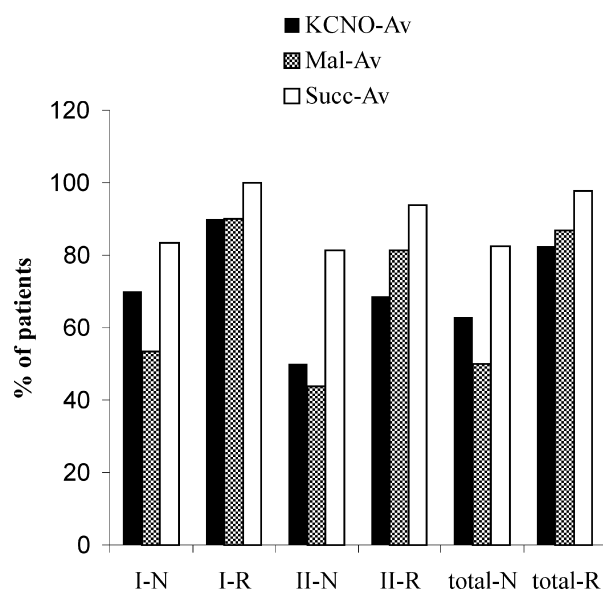


Figure 5. Results of skin prick tests of patients allergic to mugwort pollen with allergoids. KCNO, Mal-Av, Succ-Av. Group I did not receive immunotherapy. Group II were in the course of immunotherapy. N, no reaction to allergoid; R, reaction was reduced compared to the unmodified extract.

lated and maleylated derivatives than for the succinylated one). It is worth mentioning that a higher percentage of patients showed no reaction to KCNO-Av than to Mal-Av, but that was not the case for the ability of derivatives to elicit a reduced reaction (columns designated as R in Fig. 5). The major difference of the succinylated derivative from other derivatives was noted for the group of most sensitive patients, designated as 5+ using a histamine grading system (Fig. 6); and 83% of patients showed no reaction to Succ-Av when compared to the value of 28% to KCNO-Av or 22% to Mal-Av.

Discussion

Subcutaneous specific IT is considered to be effective in treating respiratory allergies (33). The main concern about subcutaneous IT has always been its safety; in fact, the risk of severe or fatal adverse reactions makes its use questionable (34). These safety issues have stimulated the search for new and safer routes for administering IT, such as chemical modification of allergens.

The principal approach to is to modify B-cell allergen epitopes in order to prevent IgE binding and effector-cell cross-linking while preserving T-cell epitopes. In this way, the modified allergen will be directed to T cells by a phagocytosis/pinocytosis-mediated antigen uptake mechanism, bypassing IgE cross-linking and IgE-dependant antigen presentation (35).

An important goal was to develop derivatives with the same molecular size as the native form to use in local

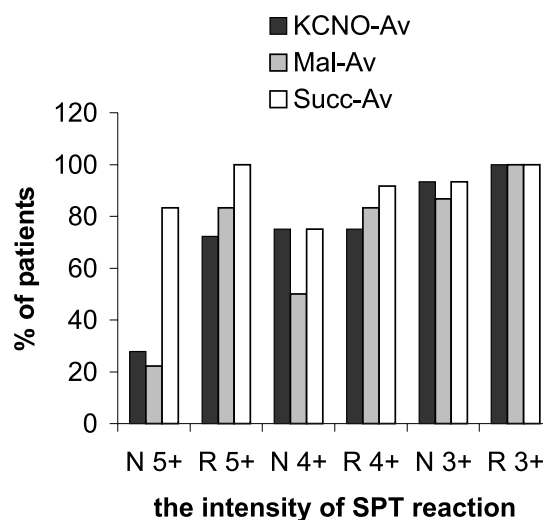


Figure 6. Results of skin prick tests (SPT) of patients allergic to mugwort pollen with allergoids. N, no reaction to allergoid; R, reaction was reduced compared to the unmodified extract. 5+, group of patients with high skin reactivity; 4+, group of patients with moderate skin reactivity; 3+, group of patients with low skin reactivity.

administration. Carbamylated and acid anhydride derivatives fulfilled such criteria. This study addressed the allergenic properties of different low-molecular-weight derivatives to determine whether or not acid anhydride derivatives had similar clinical potency to carbamylated ones.

The first step was to investigate the biochemical properties of derivatives, regarding the extent of modification. Both strategies used reaction with the ϵ -amino group of lysine, which was converted into an uncharged ureido group (KCNO-Av derivative), a negatively charged maleyl-group (Mal-Av derivative), or a negatively charged succinyl group (Succ-Av). According to the degree of amino-group substitution determined by the TNBS reagent, both procedures gave approximately the same value of conversion (more than 85% for all investigated derivatives). The results obtained in our study are in agreement with the extent of modification obtained by Mistrello et al. for *Parietaria judaica* pollen extract or well-defined allergens such as ovalbumin or Par j 1 (17). The influence of modification on enzyme activity was the same order of magnitude in all derivatives. The changes in pI values demonstrated in IEF, showed that the acid anhydride derivatives were more acidic when compared to carbamylated ones (Fig. 1A). The same phenomenon of higher acidity of maleylated than succinylated derivatives was observed for *Dactylis glomerata* allergoids. These experiments showed that all three derivatives had comparable physicochemical properties regarding the extent of modification (in particular, the same degree of amino-group conversion). The experiments performed on animals confirmed that all three types of low-molecular-weight

allergoids were able to induce IgG-antibody responses. The antibodies obtained to modified proteins were able to recognize unmodified proteins (Fig. 4). The main conclusion from these findings is that not only were modified epitopes responsible for eliciting IgG response, but also unmodified ones, which could be of significance to future IT for blocking IgG antibodies in allergic patients.

Another important concern about acid anhydride derivatives is their susceptibility to acid hydrolysis (36,37). Although they are quite stable in a neutral environment, the chemistry of the bond formed in the derivatives allows the possibility of limited hydrolysis in the highly acidic environment of the patient gut if the allergen is given via the oral route or during antigen processing in the acidic environment of lysosomes. The half-life of ϵ -maleyllysine is approximately 11 h at pH 3.5 and 37°C (35). Such a property could possibly represent an advantage of these derivatives, which may bypass the mouth and possible side effects like oral/sublingual itching and lip edema, and then slowly release the native allergen in the acidic environments.

The immunoblot experiment of IgE and IgG binding showed that although the same degree of conversion of amino groups was achieved in all derivatives, modified epitopes containing negatively charged groups were less susceptible to human IgE binding than the epitopes containing ureido groups (Fig. 3A). We conclude that the conformational changes were greater than in the case of carbamylation probably due to steric repulsions.

In the group of untreated patients (pool I; band (a) in Fig. 3B), and the group of treated patients (pool II; band (b) of Fig. 3B) the majority of IgG-binding bands were virtually the same. The only differences were new binding bands with molecular weights of 45 and 38 kDa, and increased band intensity of the higher-molecular-weight proteins in the carbamyl and maleyl derivatives (band (b) in Fig. 3B). Because immunoblot is only a semiquantitative method we could not discuss the possible effect of IT on the level of mugwort-specific IgG. In general, the inducing of IgG to new epitopes, as seen in the immunoblot, in mugwort pollen is not of a high degree. This might be due to imperfections in the method itself when native epitopes are in question. The denaturing effect of SDS-PAGE and mercaptoethanol on the native epitopes of proteins may cause the lack of new bands in the treated patients' pool. The same effect was demonstrated for rabbit IgG- and human IgE-binding epitopes of Art v 2 allergen (32).

The reduction of IgE binding was shown quantitatively in an ELISA inhibition assay, where the succinylated derivative showed the most reduced IgE-binding properties (Fig. 2).

Tests of skin reactivity to allergoids showed that slight changes in the chemical nature of the group that derivatised the amino group of lysine could influence skin reactivity. The different results from immunoblots and SPT could arise from the differences in IgE binding to sequential epitopes (in denatured or partly renatured proteins on the nitrocellulose membrane) or conformational epitopes (in native proteins). Patients were able to distinguish between one more double bonds in the maleyllysine group compared to the succinyllysine group. In both groups of patients the maleylated allergoid showed properties similar to the carbamylated derivative (Fig. 5). The succinylated derivative turned out to be the best preparation, especially because of its low allergenicity in the group of highly allergic patients (Fig. 6) regardless of their IT status. The properties of all three derivatives were equally good in the group of patients with moderate or low skin reactivity (Fig. 6). This study provides good scientific support for the clinical use of succinylated and maleylated allergens in local IT trials. An additional feature arising from the results presented here is a much greater ability of carbamylated allergens to recognize a patient's IgG, to an extent similar to unmodified proteins—leading us to conclude that the carbamylated sample might have an optimal ratio of reduced allergenicity and retained immunogenicity.

In the light of the results presented here, the success of IT trials conducted with carbamylated allergoids is based on their ability to recognize a patient's IgG. It is reasonable to expect the same protecting effect from modifying the ϵ -amino group of lysine in acid anhydride derivatives to enzymatic degradation, as seen for carbamylated derivatives (19). Therefore, it would be preferable to investigate the pharmacokinetics of these derivatives in local IT preparations, regarding the possibility of limited hydrolysis of these derivatives before any clinical trial.

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