Artemisia vulgaris pollen allergoids digestibility in the simulated conditions of the gastrointestinal tract

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Abstract: Chemically modified allergens (allergoids) have found use in both traditional and novel forms of immunotherapy of allergic disorders. Novel forms of immunotherapy include local allergen delivery, *via* the gastrointestinal tract. This study conveys the gastrointestinal stability of three types of mugwort pollen allergoids under simulated conditions of the gut. Allergoids of the pollen extract of *Artemisia vulgaris* were obtained by means of potassium cyanate, succinic and maleic anhydride. Gastrointestinal tract conditions (saliva, and gastric fluid) were simulated in accordance with the EU Pharmacopoeia. The biochemical and immunochemical properties of the derivatives following exposure to different conditions were monitored by determining the number of residual amino groups with 2,4,6-trinitrobenzenesulfonic acid, SDS PAGE, immunoblotting and inhibition of mugwort-specific IgE. Exposure to saliva fluid for 2 min did not influence the biochemical and immunochemical properties of the derivatives. In the very acidic conditions of the simulated gastric fluid, the degree of demaleylation and desuccinylation, even after 4 h exposure, was low, ranging from 10 to 30 %. The digestion patterns with pepsin proceeded rapidly in both the unmodified and modified samples. In all four cases, a highly resistant IgE-binding protein the Mw of which was about 28 – 35 kD, was present. Within the physiological conditions, no new IgE binding epitopes were revealed, as demonstrated by immunoblot and CAP inhibition of the mugwort specific IgE binding. An important conclusion of this study is the stability of the modified derivatives in the gastrointestinal tract of patients, within physiological conditions. The means that they are suitable for use in much higher concentrations in local forms of immunotherapy than unmodified ones.

Keywords: allergoid, *Artemisia vulgaris*, mugwort pollen, chemical modification, digestion, maleic anhydride, potassium cyanate, succinic anhydride.

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Abbreviations: SF (simulated saliva fluid), SGF (simulated gastric fluid), Av (*Artemisia vulgaris* = mugwort pollen extract), M-Av (maleylated derivatives of the mugwort pollen extract), S-Av (succinylated derivatives of the mugwort pollen extract), C-Av (carbamylated derivatives of the mugwort pollen extract), PBS (phosphate buffered saline), SLIT-sublingual local immunotherapy.

INTRODUCTION

Various forms of chemically modified allergens, or genetically engineered recombinant allergens have been developed[1,2] in order to improve the safety and efficiency of conventional specific immunotherapy.

The principal aproach to allergen modification is to modify B cells 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-dependent antigen presentation.[3] Thereby, all side-effects of the IgE cross-linking by an allergen on the effector cells of the immune system, such as histamine and mediator release, would be diminished in the case of allergoid immunotherapy, while the protective immune response *via* the preserved T helper (Th) cell epitopes would be enhanced during the course of immunotherapy.[4]

Previous forms of chemically modified allergens were of high molecular weight[2,3] and were unable to pass mucosal barriers and reach the immune system of patients when applied *via* local routes.

Low-molecular weight (LMW) derivatives of allergens are modified allergens of the same (native) size as unmodified ones, with the potential to be used in local forms of immunotherapy.[5–7] They can be obtained by carbamylation[8–10] and treatment by maleic or succinic anhydrides.[11,12] Moreover, the conversion of allergens into highly negative (acidic) derivatives could direct them to scavenger receptors (SRs), specific for highly negative molecular species, modulating the immune response towards the Th1 type.[13] This type of immune modulation was shown for some maleylated or acetylated proteins and peptides.[14] Therefore, the maleylated allergens can be considered as having a potential immunostimulatory effect on the immune system. Their mode of action would not only be the reduction of an immediate phase response, but would also be effective at the T cell level.

All three types of LMW allergoids showed reduced allergenicity in the skin prick testing of allergic patients.[12] A study of the pharmacokinetics of carbamylated allergoids suggested that these derivatives might be protected against proteolytic cleavage, and showed that they attained even higher levels in blood samples after bucal administration than the unmodified proteins.[15]

In a previous study[16] incomplete hydrolysis of mugwort pollen allergens and allergoids were observed under simulated gastrointestinal conditions, while retention of the IgG binding was monitored in an ELISA (enzyme linked immunosorbent assay), after exposure to the acidic conditions of the gut. Allergoids, particularly those derived from pollen extracts, have been successfully established as prepara-

tions for subcutaneous injection immunotherapy. A surge of interest in local routes of immunotherapy, particularly sublingual/oral, raises the question as to the suitability of allergoids for this application. Stability under the various conditions encountered in the digestive tract is one consideration. Hence, the aim of this study was to investgate the susceptibility of various *Artemisia vulgaris* allergoids to degradation.

RESULTS

The effect of saliva fluid on the allergen and allergoids of mugwort pollen extract

Exposure to the simulated saliva fluid for 2 min did not influence the biochemical and physicochemical properties of the allergen and allergoids, as demonstrated by the measurement of the residual amino groups by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method[17] and the protein pattern by SDS PAGE and IEF (results not shown). Although the results were negative, this feature of the modified derivatives is beneficial from the point of view of local delivery (sublingual).

The effect of the acidic pH of the simulated gastric fluid on the allergen and allergoids of Av

The effect of the acidic conditions on the acylated derivatives, which are prone to acid hydrolysis, was monitored by measuring the number of amino groups by
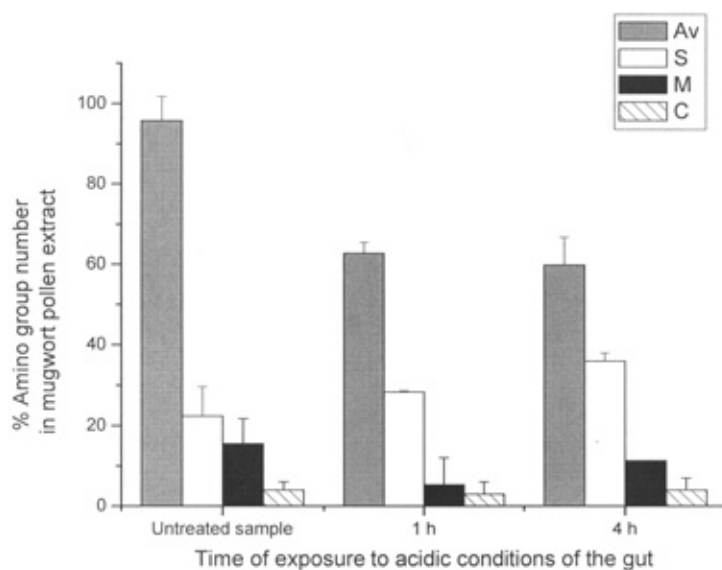

Fig. 1. Determination of the amino group number in mugwort pollen extract (Av) and allergoid samples of maleylated proteins (M), succinylated proteins (S), carbamylated proteins (C) exposed to the acidic conditions of the SGF (without the addition of pepsin) for one and four hours. The amino groups were quantified by the TNBS method. The number of amino groups in the unmodified (Av) sample was regarded as 100 % of the amino group number, while the number of amino groups determined in the modified samples were calculated relative to the unmodified sample (in % of the control, unmodified sample). The protein modification which acts on the amino group leads to the decrease in the detected amino group number.

the TNBS method (Fig. 1). The Av sample demonstrated a decrease in the number of free amino groups, presumably due to denaturation of the proteins in the very acidic conditions, leading to aggregation and precipitation, while a slight increase in the number of free amino groups was registered in the case of the succinyl derivative. With the carbamylated and maleylated derivatives, the number of free amino groups did not change significantly. With the maleylated sample, one-hour hydrolysis led to a reduction in the number of amino groups which intensified with prolongation of the exposure, probably, in part, due to the effect of denaturation. The extents of demaleylation and desuccinylation, even after 4 h exposure, were very low, ranging from 10 to 30 %.

Digestion pattern under simulated gastric conditions

Prolonged exposure of the allergens of Av to simulated gastric conditions (for 1 and 4 h), leads to rapid proteolysis of the proteins by pepsin (Fig. 2). The pattern seen after 30 s of exposure was the same as after 1 h (results not shown). A highly resistant protein, Mw of about 28 to 35 kD, could be seen in the Av extract and the KCNO treated proteins. The digested maleylated and succinylated proteins gave hardly visible peptides, Mw of about 5–15 kD. The majority of the allergens in the Av and carbamoylated samples were fully degraded by pepsin. The acidic deriva-
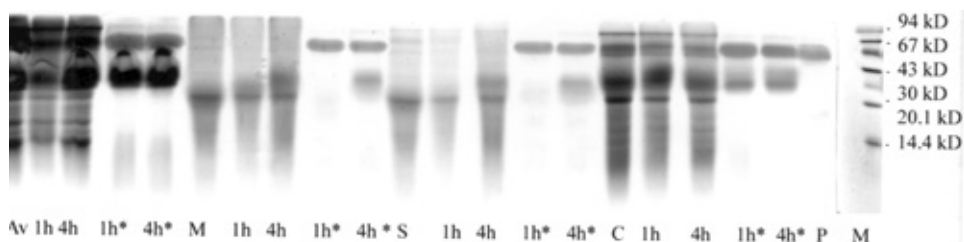

Fig. 2. SDS PAGE on 18 % gel of mugwort pollen proteins and allergoids exposed to the acidic conditions of the gut for 1 and 4 h and to the simulated gastric fluid for one (1h*) and 4 h (4h*), applied in sequence after the appropriate controls: mugwort pollen extract (Av), maleylated proteins (M), succinylated proteins (S), carbamylated proteins (C): P – pepsin control; M – molecular weight markers. The proteins were visulized by Coomassie Brilliant Blue R250 staining.

tives (M-Av and S-Av) appeared apparently more susceptible to pepsin digestion (the samples treated for 1 h with SGF). After prolongation of the exposure to the gastric conditions, and probably through the effect of deamidation at very acidic pH, the resistant protein bands also appeared in the modified samples. The explanation could be that the dye binding properties of the allergens changed when they became highly acidic. An acidification of the proteins in all three allergoid preparations led to a lower dye binding, when compared to the ummodified proteins, due to a blocking reaction on the lysine residual group.[18,19]

The effects of acidic conditions and the SGF on IgE binding properties of allergens and allergoids in immunoblot

The proteins of the Av allergen and the allergoids were digested in the simulated gastric conditions and resolved by SDS PAGE. In the immunoblotting with the sera of the allergic patients it was shown that the SGF resistant proteins bind IgE from the patients' sera (Fig. 3). Although to a lower extent, the C-Av derivative behaves in a smilar way, with retention of the 30 kD IgE binding band. The exposure time does not influence the intensity of the band. The derivatives treated with
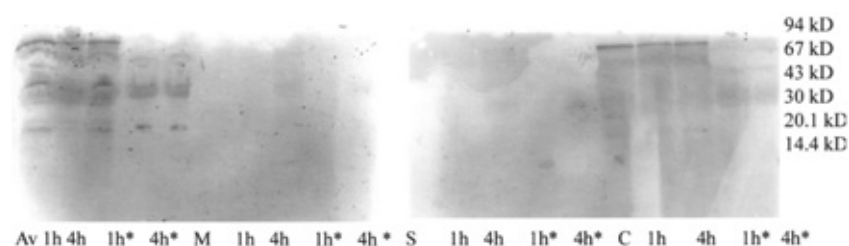

Fig. 3. Determination of the IgE binding bands on a nitrocellulose membrane after SDS PAGE on an 18 % gel of mugwort pollen extracts and allergoids, exposed to the acidic conditions of the gut for 1 and 4 h, and to the simulated gastric fluid for one (1h*) and 4 (4h*), applied in sequence after the appropriate controls: mugwort pollen extract (Av), maleylated proteins (M), succinylated proteins (S), carbamylated proteins (C). The resolved proteins were probed with the allergic patients' sera and bound IgE was detected with monoclonal anti IgE antibody alkaline phosphatase labeled. The bands were visualized using a precipitating substrate for alkaline phosphatase (BCIP/NBT), as described in the experimental.

acid anhydrides completely lacked the IgE binding bands. Digestion with SGF does not influence the pattern of IgE binding, but acidic conditions do. After 4 h exposure to gastric conditions, a scarcely visible band of the same weight appeared in both the S-Av and M-Av derivatives, regardless of whether treated with SGF, or just exposed to acidic conditions.

CAP inhibition

The effect of the simulated *in vitro* conditions were quantified by CAP inhibition (Fig. 4). A pool of the patients' sera was preincubated with allergens, allergoids or control proteins, and the residual mugwort pollen specific IgE was quantified by UniCap. Saliva fluid exposure did not influence the IgE binding in any case. There appeared to be a high percentage of residual IgE binding activity in the Av extract, after exposure to SGF (the decrease seen was slightly over 20 %), due to the high IgE binding potential of the residual allergens, which correlated with the binding pattern seen in the immunoblot (Fig. 3). Presumably, smaller peptide fragments may also inhibit the binding of the mugwort specific IgE. SGF exposure of the maleylated sample led to a small decrease in the IgE binding, while the IgE binding potential of the succinylated sample did not change on digestion. The carbamylated sample mostly resembled the unmodified sample, except that IgE
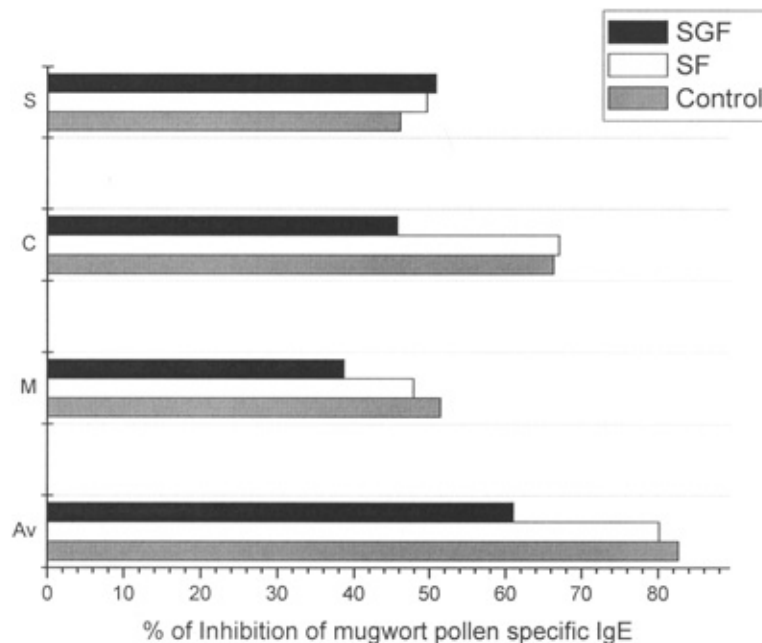
Fig. 4. Determination of IgE binding potencies of mugwort pollen extract (Av) and allergoids (maleylated proteins (M), succinylated proteins (S), carbamylated proteins (C)) treated with saliva fluid (SF), simulated gastric fluid (SGF) by means of CAP inhibition of the mugwort pollen specific IgE.

binding potential was lower, which was also the case with acyl derivatives, when compared to the unmodified extract, in any case under the examined conditions (untreated samples, samples treated with acidic conditions and samples exposed to SGF conditions). The results obtained by CAP inhibition are in accordance with the liberation of a small percent of amino groups in acidic conditions (Fig. 1). Presumbly, as there was no significant deamidation in the modified Av derivatives (Fig. 1), there was no significant regeneration of IgE binding epitopes (demonstrated in the immunoblot and CAP inhibition of the IgE binding to the mugwort disc). This is an important finding from the aspect of allergoid safety in local immunotherapy trials.

DISCUSSION

Local allergen delivery has lately become an important route of specific immunotherapy administration. Modified derivatives of allergens of native molecular size have also been introduced in immunotherapy of allergic diseases in adults[5, 7] and children.[8] Acid anhydride derivatives were also considered to have a potential for use in this form of immunotherapy.[12]

A modified derivative introduced through local routes may finally reach patients' gastrointestinal tract and become exposed to very different environments. A

protective effect on the digestion by a chemical modificiation might occur,[17] as well as a limited hydrolysis, leading to revealing of some IgE binding epitopes by exposure to (especially) acidic conditions of the gut.

It has been demonstrated that saliva fluid does not influence the allergenic properties of native and modified derivatives. In sublingual forms of SLIT, a 2 min absorption through the mucosa will not change the properties of the derivatives introduced. This is an important finding, considering possible side-effects of SLIT on the mouth, such as itching and edema.[2,20] The demonstrated stability of the derivatives tested can be of importance in local allergoid delivery.

The derivatives finally reach the patent's gut and the effects of exposure to these conditions was affected by the chemical modifications, although to a less extent than expected. By monitoring the course of ε-amino group regeneration in a highly acidic environment, it was noticed that no significant deamidation occurred (Fig. 1). The deamidation reaction occurred to no more than 30 % of the sample during exposure for one hour. Prolongation of the exposure did not facilitate the process. According to the presented results, it was not possible to demonstrate any significant protective effect of amino group modification from the digestion pattern with pepsin (Fig. 2).

Contrary to a general belief that pollen extracts are quite labile to proteolysis,[21,22] the presence of protein bands at about 28 – 35 kD were evidenced in the mugwort pollen extract treated with pepsin. These resistant *Artemisia vulgaris* proteins contain intact IgE-binding epitopes and several potential enzymes cut sites which are protected from the enzyme, probably by the compact structure of the protein and/or glycosylation. This is the first report of pepsin resistant proteins from a pollen source. Therefore, hitherto, there were no data on the clinical significance of these findings on the higher incidence of gastrointestinal side-effects during local immunotherapy to the mugwort extract. However, in the case of an allergen source which is resistant to pepsin-digestion, modified derivatives of reduced capacity to bind specific IgE could be recommended. According to the presented results, the IgE binding is not restored in the modified derivatives after exposure to simulated gastric conditions (Figs. 3 and 4). Therefore, they could be regarded as a safe immunotherapy option for application in local allergen delivery.

EXPERIMENTAL

Allergen and allergoids preparations

The pollen samples, obtained from the Institute for Immunology and Virology, Torlak, Belgrade, Serbia, were collected from mugwort weed collected in the outskirts of Belgrade. The pollen extracts, as well as the modified derivatives, were prepared as described previously.[12] A mugwort pollen extract (10 mg of proteins) was modified by KCNO treatment according to Mistrello *et al.*[8] The modified sample, designated C-Av, was dialyzed against water and concentrated to a protein concentration of 1 mg/ml. The same protein quantities were used in the modifications with 400 mM succinic or maleic anhydride, according to a previously described procedure.[12] The final samples, designated M-Av and S-Av, were dialyzed against water and lyophilized. The protein concentration was determined by the Bradford method.[23]

Determination of amino groups

The number of amino groups in the control and modified samples, after exposure to the acidic conditions of the gut, without the addition of pepsin, was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS) as the reagent according to a previously described procedure.[17] The results are expressed as means of three different determinations and for the modified samples as a percentage of the number of amino groups determined for the unmodified sample (expressed as 100 %).

Digestion of samples in SF and SGF

Digestion of samples in SF and SGF was performed as already described.[16]

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

SDS PAGE was carried out according to Laemmli[24] using a Hoefer Scientific Instrumentation apparatus with a discontinuous buffer system. Native and modified samples in the same quantity of 10 μg per well (Av, C-Av, S-Av and M-Av) were diluted in sample buffer (5 % β-mercaptoethanol) and boiled for 5 min before the run. The protein components were resolved on an 18 % gel, with a standard protein mixture for mol. wt. reference, at 80 V for 1 h and 250 V for a further 2 h. The gels were stained with Coomassie Brilliant Blue R-250 to visualize the separated proteins.

Resolved components were blotted by a semi-dry electroblotter onto nitrocellulose paper. The transferred proteins were blocked in TBS – 0.1 % Tween 20 for 1 h and dried until development.

Human sera

The sera from 25 patients allergic to mugwort pollen, with a documented clinical history of mugwort pollen allergy and with no recorded immunotherapy to mugwort pollen, were pooled for the CAP inhibition experiment. The patients were allergic to mugwort pollen, with documented clinical histories of allergic rhinitis, allergic asthma, allergic rhinoconjunctivitis and allergic conjunctivitis in accordance with the Position Paper for the revised nomenclature for allergy.[25] Sera from five non-atopic persons were pooled and used as a control.

IgE detection

IgE 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 in a diluting buffer (TBS – 0.1 % BSA). As secondary antibodies, alkaline phosphatase labelled monoclonal anti human IgE (Sigma Chemical Co., St Louis MO, USA) was used. The binding patterns were visualized with a substrate solution consisting of 1.5 mg BCIP (5-bromo-4-chloro-3-indolyl phosphate) and 3 mg NBT (nitroblue tetrazolium) in 10 mL of 100 mM Tris buffer, containing 150 mM NaCl, and 5 mM $MgCl_2$, pH 9.6.

CAP inhibition of IgE binding from a pool of allergic patients sera

For CAP inhibition, samples of mugwort pollen extract, maleylated, succinylated and carbamylated allergoids were diluted in PBS to a concentration of 20 μg/mL. The saliva (2 minutes treated samples) and SGF (1 hour treated samples) exposed samples were diluted to the same volume, as were the untreated proteins. As controls, the human serum pool was incubated with saliva or SGF solutions diluted to the same volume as the samples and ovalbumin in the same concentration as the samples, or with PBS alone. Human serum from subjects with skin tests negative to mugwort pollen extract was also used as a negative control. After overnight incubation, the IgE inhibiton was determined by a UniCap (Pharmacia, Sweden) according to the manufacturer's instructions. The percent of inhibition was calculated as described previously[26] and from the appropriate positive control.

ИЗВОД

ДИГЕСТИБИЛНОСТ АЛЕРГОИДА ПОЛЕНА ПЕЛИНА У СИМУЛИРАНИМ УСЛОВИМА ГАСТРОИНТЕСТИНАЛНОГ ТРАКТА

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У овом раду су приказани резултати испитивања стабилности три типа алергоида полена пелина у симулираном желудачном соку. Користећи калијум-цијанат, анхидрид ћилибарне и анхидрид малеинске киселине, направљени су алергоиди полена пелина (*Artemisia vulgaris*). Салива и желудачни сок су симулирани на основу европске фарма-копеје. Биохемијске и имунохемијске особине деривата, после излагања различитим условима, праћене су: одређивањем броја слободних амино група у реакцији са TNBS, SDS PAG електрофорезом, имуноблотом и одређивањем пелин-специфичног имуно-глобулина E (IgE). Излагање сланови у трајању од 2 минута не утиче на биохемијске и имунохемијске особине деривата. У киселој средини желудачног сока не долази до значајног демалеиловања и десукциниловања. Чак и после четворочасовног излагања, тај проценат је у опсегу 10–30 %. Алергоиди пелина се тренутно дигестују пепсином, са изузетком високо резистентне протеинске траке молекулске масе 28–35 kD, која одго-вара важном IgE-везујућем протеину полена пелина. Имуноблотом и CAP-инхибицијом је показано да, у оквиру физиолошких услова, не долази до стварања нових IgE-ве-зујућих епитопа. Хемијска стабилност модификованих деривата у симулираним усло-вима желудачног сока омогућује да се током имунотерапије могу примењивати веће дозе алергоида него немодификованог екстракта полена пелина.

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