IgG binding of mugwort pollen allergens and allergoids exposed to simulated gastrointestinal conditions measured by a self-developed ELISA test

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Abstract: This study considers the influence of exposure to simulated gastrointestinal conditions (saliva, gut, intestine and acidic conditions of the gut) on IgG binding of unmodified allergens and three types of LMW allergoids of *Artemisia vulgaris* pollen extract obtained by means of potassium cyanate, succinic and maleic anhydride. It also concerns the optimization of a self-developed ELISA assay for comparison of the specific IgG binding of mugwort pollen extract and modified mugwort pollen derivatives. The ELISA was conducted with a mugwort pollen extract coupled to the plate, using the sera from 12 mugwort-pollen allergic patients. The exposure to saliva fluid for 2 min did not influence the IgG binding properties of allergens and allergoids. Exposure of mugwort pollen allergens and LMW allergoids to the acidic conditions of the gut did not dramatically change their IgG binding properties. By exposing mugwort pollen extract and LMW derivatives to the SGF conditions for 1 h, the percent of IgG binding epitopes was reduced to a half of its starting value in the extract and to about 30 % in all the allergoid samples. After prolonged exposure only the carbamyl derivative showed reduced IgG binding. Changes of the IgG binding potential of all four samples after exposure in SIF followed a similar pattern.

Keywords: simulated gastrointestinal conditions, *Artemisia vulgaris*, mugwort, pollen, allergoid.

INTRODUCTION

Different forms of local immunotherapies of allergic diseases[1–4] as well as chemical modification of allergens were mainly considered to be a safe way of administering allergenic extracts. In local forms of immunotherapy, some low-molecular weight allergoids could also find application. Low-molecular weight (LMW) derivatives of allergens are modified forms of allergens of the same (native) size as the unmodified ones. Two

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prinicpal approaches were undertaken to obtain LMW allergoids: carbamylation[5–7] and acid anhydrides treatment.[8,9] Such derivatives reduce the allergenicity while preserving the ability to induce blocking of IgG antibodies, whose increased level during allergen-specific immunotherapy correlates well with clinical improvements.[10] Thus, the retention of IgG-binding epitopes on the modified allergens is of significance in the protection against harmful effects of IgE-mediated allergic reactions that could appear during local allergen delivery throughout the gastrointestinal tract of an allergic person, especially because it has been shown that there is a fraction of IgG antibodies in mucosal secretions that contributes to the immunity of mucosa, besides IgA antibodies.[10]

A study of the digestion stability of birch, timothy, ragweed and mugwort pollen extracts showed a remarkable difference among the investigated allergenic extracts. Mugwort pollen extract was shown to be the source of the most resistant allergens. A double-band fraction of 28 – 35 kD was isolated from mugwort pollen which was a pepsin-resistant allergen.[11] Moreover, its prolonged and limited digestion in the gut could raise the question of higher incidence rates of side-effects during local immunotherapy trials. Regarding this, a better knowledge of how the mugwort pollen derivatives behave in simulated conditions of the gut, might be important in optimizing the dosage of the extracts used in local forms of immunotherapy conducted with allergenic extracts rich in these pepsin-resistant allergens.

This study considers the dependence of exposure to simulated *in vivo* gastrointestinal conditions on the IgG binding of three types of LMW allergoids of *Artemisia vulgaris* pollen extract. Although all three reactants act on the ε-amino group of Lys, the bonds formed in such a way differ in chemical stability as well as stereochemistry[12] and yield quite different modification products. A self-developed ELISA assay was also optimized to compare the specific IgG binding of untreated mugwort pollen extract and modified mugwort pollen derivatives.

RESULTS AND DISCUSSION

Human sera: The characteristics of the group of patients included in the study (8 men and 4 women; aged 28 – 55) are shown in Table I. For the pool used in the ELISA testing, the values for the total and specific IgE were determined to be: 586 kUA/l and 12.8 kUA/l, respectively.

IgG binding: The values obtained for the IgG binding of untreated samples were in the order: mugwort pollen sample, carbamyl derivative, maleyl derivative and succinyl derivative. The values were in agreement with published observations that the succinyl derivative binds IgG from the pool of the sera of allergic patients to a lesser extent than the other samples tested. According to the majority of the assays included in a previous study, the succinyl modification gave derivatives with the most changed IgE and IgG binding properties when compared to the native sample.

The influence of saliva exposure. The exposure to saliva fluid for 2 min did not influence the IgG binding properties of allergens and allergoids, as demonstrated by measuring the residual IgG binding in the samples (Figs. 1–4). In sublingual forms of IT, 2 min absorption through mucosa did not change the properties of the derivatives introduced. With
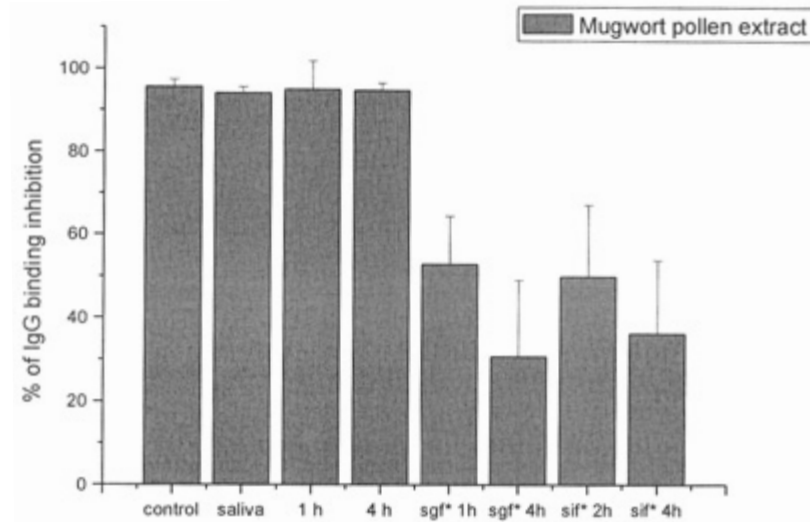
Fig. 1. ELISA inhibition of IgG binding for mugwort pollen extract before and after simulated gastrointestinal exposure. Control-non treated mugwort pollen proteins; saliva-saliva treated sample; 1 h-sample after 1 h treatment with the acidic conditions of the gut; 4 h-sample after 4 h treatment with the acidic conditions of the gut; sgf*-simulated gastric juice treated sample; sif*-simulated intestine juice treated sample; 1 h, 2 h and 4 h-duration of the treatment.
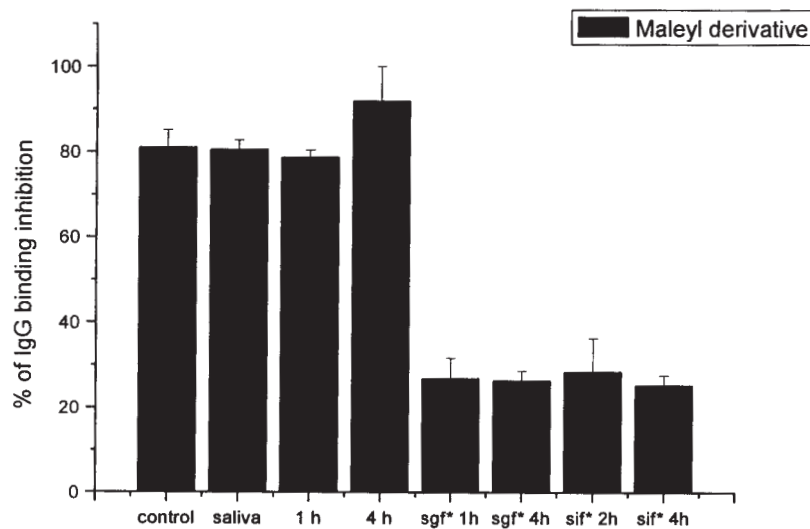

Fig. 2. ELISA inhibition of IgG binding for maleyl derivatives of mugwort pollen proteins before and after simulated gastrointestinal exposure. Control-non treated maleyl derivatives of mugwort pollen proteins; saliva-saliva treated sample; 1 h-sample after 1 h treatment with the acidic conditions of the gut; 4 h-sample after 4 h treatment with the acidic conditions of the gut; sgf*-simulated gastric juice treated sample; sif*-simulated intestine juice treated sample; 1 h, 2 h and 4 h-duration of the treatment.

respect to the side-effects of SLIT on the mouth (itching and oedema),[1,13] the stability of the derivatives tested is very important.

The influence of exposure to the acidic conditions of gastric fluid. According to the percent of residual IgG binding, after 4 h of gastric exposure, the acyl derivatives start to hydrolyze (Figs. 2 and 3), liberating IgG epitopes blocked by the modification reaction. The carbamyl derivative did not change its IgG binding potential in the very acidic conditions of the gut environment (Fig. 4).

The influence of exposure to gastric fluid. On exposing the mugwort pollen extract (Fig. 1) and the low-molecular derivatives to simulated gastric fluid (SGF) conditions for 1 h (Figs. 2–4), the percent of IgG binding epitopes was reduced to half of its starting value. In all the samples, the residual protein remained fully resistant to digestion and contributed to IgG binding. Its IgG binding potential was still below the value of the native extract, however, a significant proportion of almost 75 % of the IgG binding epitopes remained in the modified derivatives when compared to the native sample.

TABLE I. Patient's clinical characteristics

Patient's No.	Sex	Current age/y	Diagnosis	Prick testing with mugwort pollen (weal/flare [mm])
1	F	27	Allergic rhinoconjunctivitis	6/20
2	F	53	Pollinosis	7/15
3	M	53	Pollinosis	4/7
4	F	30	Allergic rhinitis	10/20
5	M	32	Pollinosis	8/11
6	F	49	Allergic rhinitis	7/10
7	M	49	Allergic rhinitis, Laryngitis	8/18
8	M	55	Allergic rhinitis	5/8
9	M	48	Pollinosis	6/9
10	M	32	Allergic rhinitis	5/10
11	M	30	Pollinosis	4/10
12	M	57	Allergic rhinitis	8/13

The influence of exposure to intestine fluid. Although exposure to SIF conditions significantly reduces the allergenic potential of the derivatives,[11] presumably by digestion of the pepsin resistant mugwort pollen allergen, the IgG binding potential after 2 h exposure was similar to the SGF treated samples after 1 h (Figs. 1–4). A portion of the residual peptide fragments contributed to the extent of inhibition.

Pollen allergens are generally believed to be labile under the conditions of the gut environment. Some earlier reports suggest rapid degradation of grass and tree pollen proteins in the gut and duodenal fluids.[14–17]
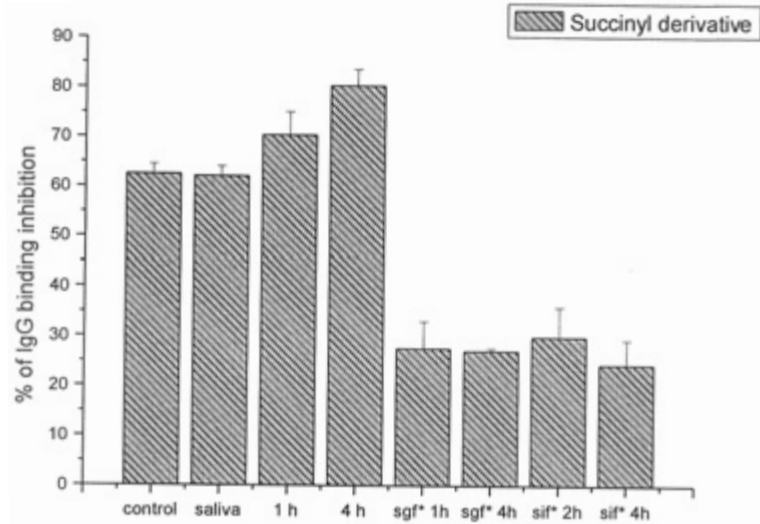
Fig. 3. ELISA inhibition of IgG binding for succinyl derivatives of mugwort pollen proteins before and after simulated gastrointestinal exposure. Control-non treated succinyl derivatives of mugwort pollen proteins; saliva-saliva treated sample; 1 h-sample ofter 1 h treatment with the acidic conditions of the gut; 4 h-sample after 4 h treatment with the acidic conditions of the gut; sgf*-simulated gastric juice treated sample; sif*-simulated intestine juice treated sample; 1 h, 2 h and 4 h-duration of the treatment.
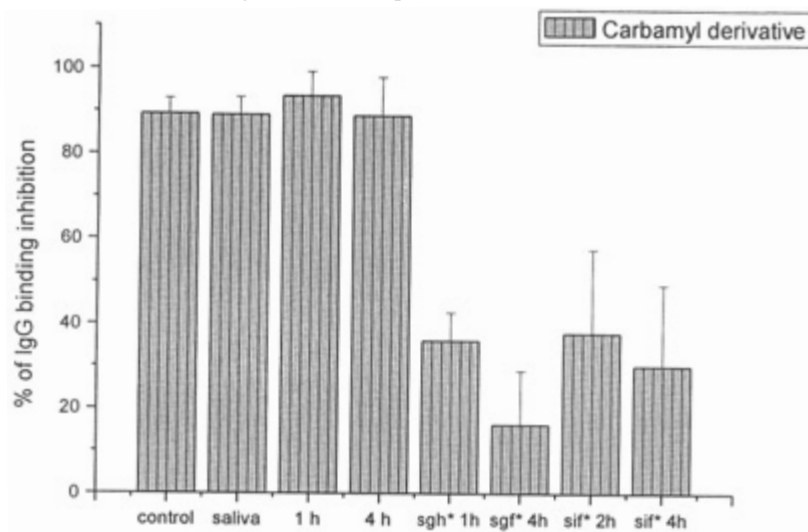

Fig.4. ELISA inhibiton of IgG binding for carbamyl derivatives of mugwort pollen proteins before and after simulated gastrointestinal exposure. Control-non treated carbamyl derivatives of mugwort pollen proteins; saliva-saliva treated sample; 1 h-sample after 1 h treatment with the acidic conditions of the gut; 4 h-sample after 4 h treatment with the acidic conditions of the gut; sgf*-simulated gastric juice treated sample; sif*-simulated intestinge juice treated sample; 1 h, 2 h and 4 h-duration of the treatment.

Regarding the observation that not all of the pollen allergens degrade rapidly[10] and possible immunotherapy trials of acid anhydride derivatives, it was important to investi-

gate the IgG binding properties of these derivatives in the simulated environments of patients' saliva, gut and intestine, in order to achieve a fine tuning of the local immunotherapy dosage of these derivatives.

This study showed that the retention of IgG binding epitopes in the modified derivatives is greater than that of the unmodified sample. One of the reasons might be a reduced hydrolysis of the derivatives in the acidic conditions of the gut (as seen after 4 h exposure), but also the modifications might protect the derivatives against proteolitic cleavage. Additionally, the effect of deacylation on the extent of IgG binding is of greater magnitude when compared to the IgE binding of the same samples.[11] As most of the proteins both in the native and modified samples are degraded under SIF conditions, the blocking effect is more pronounced regarding the simulated intestinal conditions, presumably because of the protecting effect on the ε-amino group of Lys, the target of trypsin, which is the main component of pancreatin juice.

EXPERIMENTAL

Allergen and allergoids preparations. Pollen extracts as well as the modified derivatives were prepared as described previously.[8,9,18] The carbamyl derivative was prepared according to Mistrello *et al.*[5] The protein concentrations were determined by the Bradford method.[19]

The simulated saliva experiments. The simulated saliva fluid (SSF) consisted of 2.38 g Na_2HPO_4, 0.19 g KH_2PO_4, 8 g NaCl/l adjusted with phosphoric acid to pH 6.75. 150 µg of allergoids and unmodified samples were exposed to 150 µl SSF for 2 min at 37 ºC. After exposure the samples were immediately transferred to an ice-bath and kept frozen until analyzed.

The simulated gastric juice experiments. The simulated gastric fluid (SGF) consisted of 0.1 M HCl, 2 g/L NaCl and 3.2 g/L pepsin (Sigma). The same fluid was prepared without addition of pepsin. The allergens and alelrgoids were exposed to SGF both with and without pepsin for 1 and 4 hours in accordance with conditions described earlier.[20] The reaction was stopped by the addition of 1 M sodium bicarbonate to the pH value of 7.2.

The simulated intestine juice experiments. The simulated intestine fluid consisted of phosphate buffer pH 7.4, and 10 g/l of pancreatin (Sigma) in accordance with US Pharmacopoeia. The samples were exposed to SIF for 2 and 4 hours. The reaction was stopped by freezing the sample.

Human sera. The sera from 12 patients with a documented clinical history of mugwort pollen allergy and with a recorded course of specific immunotherapy to mugwort pollen were used. The patients had documented clinical histories of allergic rhinitis, allergic asthma, allergic rhinoconjunctivitis and allergic conjunctivitis. Sera from five non-atopic persons were pooled and used as a control. The values of the total and mugwort pollen specific IgE in the pooled sera were determined by the UniCap (Pharmacia, Uppsala, Sweden) system.

ELISA and ELISA inhibition. ELISAs were performed as described previously.[21] Briefly, 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, 50 µl of six-fold diluted pooled human sera, incubated with 50 µl of diluted allergen or allergoids (at the concentration of 4 µg/ml) in tubes for 30 min at 4 ºC, were added to the plates. The saliva, SGF and SIF exposed samples were diluted to the same volume as were untreated proteins. As a positive control, the human serum pool was incubated with saliva, SGF or SIF solutions diluted to the same volume as the samples and ovalbumin of the same concentrations as the samples or with TBS alone. For inhibition, diluted solutions of all samples were prepared with a diluting buffer consisting of TBS-0.5 % BSA. Human sera from persons with negative skin tests to mugwort pollen extract was used as a negative control. As a second antibody, anti-human IgG antibody labelled alkaline phosphatase (Sigma) was used. The absorbance values were measured at 405 nm after adjusting the background with a negative control. The percent of inhibiton was calculated in accordance with the formula previously described[21] and from the appropriate positive control.

ИЗВОД

IgG ВЕЗИВАЊЕ АЛЕРГЕНА И АЛЕРГОИДА ПОЛЕНА ПЕЛИНА ПРЕТХОДНО ИЗЛОЖЕНИХ СИМУЛИРАНИМ УСЛОВИМА ГАСТРОИНТЕСТИНАЛНОГ ТРАКТА МЕРЕНО ДОМАЋИМ ELISA ТЕСТОМ

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Предмет овог рада је испитивање промена IgG везивања немодификованих алергена полена A. vulgaris и три типа алергоида малих молекулских маса добијених третманом са калијум-цијанатом, и анхидридима ћилибарне и мелеинске после излагања условима гастро-интестиналног тракта (салива, желудац, танко црево и кисела средина желудачног сока) и оптимизација домаћег ELISA теста за одређивање IgG везивања алергена и алергоида полена пелина. У ELISA тесту је за плочицу куплован екстракт полена пелина и коришћени су серуми 12 пацијената алергичних на пелин. Излагање саливи у трајању од 2 минута не утиче на IgG везујуће особине алергена и алергоида. Излагање киселој средини желудачног сока значајно не утиче на IgG везујуће особине алергена и алергоида полена пелина. После излагања симулираном желудачном соку алергена и деривата у трајању од 1 сата, проценат IgG везујућих епитопа у немодификованом узорку се смањује на половину почетне вредности и на око 30 % код сва три деривата. Једино се код карбамил-деривата % IgG везивања додатно смањује са продужавањем излагања SGF-у. Промене у IgG везујућем потенцијалу сва 4 узорка после излагања симулираним условима танког црева прате сличан образац.

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