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Original scientific paper

Chemical modification of Art v 1, a major mugwort pollen allergen, by *cis*-aconitylation and citraconylation

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Abstract: Art v 1 is the major allergen of mugwort (*Artemisia vulgaris*) pollen, a significant cause of hay fever all over Europe. Specific immunotherapy is the only treatment modality for allergic disease. Application of modified allergens makes the treatment safer and more efficient. In this work, two out of three (citraconic anhydride, *cis*-aconitic anhydride, 2,3-dimethylmaleic anhydride) tested anhydrides were proven to be suitable for chemical modifications of allergens. Art v 1 was modified by *cis*-aconitylation and citraconylation in order to obtain derivatives of Art v 1 that may be suitable for further immunological testing. Acylation of Art v 1 gave derivatives (caaArt v 1 and citArt v 1) with about 80 % modified amino groups. The derivatives were in the monomeric form and had dramatically reduced pI values. Both derivatives were relatively stable at neutral pH values, while the acyl groups undergo hydrolysis under acidic conditions. Modification of allergens by *cis*-aconitylation and citraconylation could be a new tool for obtaining allergoids.

Keywords: allergoid; mugwort pollen; Art v 1; chemical modification; allergen-specific immunotherapy.

INTRODUCTION

IgE-mediated allergy is a global problem affecting more then 40 % of the population in industrialized countries.[1] In contrast to symptomatic treatments, specific immunotherapy (SIT) is the only prophylactic desensitizing therapy for allergy.[2,3] SIT modifies cellular and humoral responses to allergens by driving the immune response from the T helper 2 (Th 2) towards the T helper 1 (Th 1) type and generating allergen-specific regulatory T cells that can suppress the

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responses of effector T cells, accompanied with an increase in allergen-specific antibodies of the IgG class (blocking antibodies).[4] On the other hand, the potential for local and systemic reactions has forced improvements to the traditional use of allergen extracts. The main approaches involve the generation of hypoallergenic derivatives, by chemical modification[5,6] or protein engineering of recombinant allergens,[7,8] which are aimed at reducing potentially fatal reactions to allergen administration during immunotherapy. On the other hand, altered allergens have to retain their immunogenicity, *i.e.*, recognition of the modified allergen by T cells. Finally, it would be useful to have immunogens with an inherent strong Th 1-skewing potential, which is usually obtained by the usage of an adjuvant (*e.g.*, a monophosphoryl lipid).[9]

Scavenger receptors (SR) expressed on antigen presenting cells (APC) bind a variety of polyanionic ligands, thus delivering them into the endolysosomal pathway.[10] Many proteins are known to become SR ligands when chemically modified to enhance their negative charge by alteration of the ε-amino groups of their lysine residues with acetic or maleic anhydride.[11] It was shown that maleylating protein immunogens, so that they become SR ligands, leads to a more efficient antigen presentation to T cell receptors and to a greater immunogenicity with a dominantly Th 1 type of immune response.[12,13] The main problem with usage of modified immunogens is a reduced immunogenicity as consequence of affinity loss of T cell receptors for the modified epitopes. Shakushiro *et al.*[14] showed that ovalbumin (OVA) modified to become more acidic by succinylation (Suc-OVA), maleylation (Mal-OVA) or *cis*-aconitylation (Aco-OVA) was efficiently taken up by dendritic cells (DC) *via* SR. Mal-OVA and Aco-OVA were efficiently cross--presented by DC, while cross-presentation of Suc-OVA was hardly observed. In contrast to Mal-OVA and Aco-OVA, which are prone to deacylatation in lisosomes, Suc-OVA is chemically stable under acidic conditions. As a consequence, succinyl groups inhibit ubiquitin conjugation on the lysine residues, which is important in proteasomal degradation,[15] leading to the lack of recognition by T cells through T cell receptors (TCR).

Although a clear reduction in immunogenicity was observed for many allergoids,[5,6] hitherto the approach of reversible modification of allergens with the aim of preserving immunogenicity and recognition of T-cell receptors has not been reported.

Mugwort (*Artemisia vulgaris*) pollen is an important cause of allergy in Europe. Ninety-five percent of patients with mugwort allergy are sensitized to Art v 1, the sole major allergen in mugwort pollen.[16,17]

The aim of this work was to modify chemically Art v 1 with new modifying agents with specific features, *i.e.*, the introduction of highly negative charges that may enable them to react with scavenger receptors on antigen presenting cells and their reversible modification that may improve their immunogenicity when

compared to the traditionally used chemically modified allergens. In this study, three new chemical agents were tested and the obtained allergoids were biochemically characterized. Purified Art v 1 was modified by citraconic, *cis*-aconitic and 2,3-dimethylmaleic anhydride. *cis*-Aconitylation and citraconylation of Art v 1 gave derivatives (caaArt v 1 and citArt v 1), with about 80 % modified amino groups and dramatically reduced pI values, which could make them good candidate allergoids. The stability of the bond formed enables further animal testing of these derivatives.

EXPERIMENTAL

Citraconic anhydride, *cis*-aconitic anhydride, 2,3-dimethylmaleic anhydride and 2,4,6-trinitrobenzensulfonic acid (TNBS) were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals used in this work were of analytical grade.

Acylation of Art v 1

Art v 1 was isolated from pollen extract of *Artemisia vulgaris* and purified by ion-exchange HPLC.[18] Art v 1 (1.5 mg/ml) in 4 % $NaHCO_3$ was treated with the bolus addition of 15 portions of *cis*-aconitic or dimethylmaleic or citraconic anhydride during 30 min with extensive mixing at 4 °C. The final anhydride concentration was 400 mM. After every bolus addition, the pH was adjusted to 9.0 with solid Na_2CO_3. The mixture was extensively dialyzed against phosphate buffered saline (PBS) for 20 h at 4 °C. All samples were stored at –20 °C until use.

Determination of the free amino groups

The free amino groups were determined using the TNBS method.[19] The results are expressed as the means of three different determinations for modified Art v 1 as a percentage of the number of amino groups determined for the native Art v 1 (expressed as 100 %).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), native PAGE and isoelectric focusing (IEF)

Native and modified Art v 1 were analyzed by SDS PAGE (12 % polyacrylamide gels) under reducing condition using the Laemmli method.[20] Molecular weight standards were run simultaneously. Native PAGE was realized as for the SDS PAGE but under native conditions, without the addition of SDS in the sample buffer and in the electrophoresis buffer. The protein bands were stained with Coomassie Brilliant Blue R-250.

High-performance gel filtration liquid chromatography (HPLC)

Size exclusion HPLC was performed using an Akta HPLC system equipped with a Superdex 75 PC 3.2/30 (3.2 mm×300 mm) column (Amersham Pharmacia Biotech, Sweden). Before analysis, the samples were centrifuged (20 min, 12000 g) and 10 μl of the supernatant was injected onto the column. The components were eluted with 50 mM Tris buffer pH 8.2 containing 0.2 M NaCl and 1 mM EDTA at a flow rate of 0.05 ml/min and detected at 215 and 280 nm.

Protein concentration determination

The protein concentrations of the native and modified proteins were determined spectrophotometrically at 280 nm using an extinction coefficient 640 ml mg^{-1} cm^{-1}, calculated for Art v 1 as described previously.[18]

pH stability

The pH stability of the Art v 1 derivatives was estimated by determination of the free amino groups remaining after exposure of the derivatives to PBS (pH 7.2), 100 mM acetate buffer (pH 4.5) or 100 mM phosphate buffer (pH 2.0) at 37 °C for 1, 4 and 18 h. The results are expressed as means of two different determinations for modified Art v 1 as a percentage of the number of amino groups determined for the native Art v1 (expressed as 100 %).

RESULTS AND DISCUSSION

In present study, Art v 1 was modified by adding negative charges, which should, in principle, facilitate SR-mediated uptake and presentation of this allergen by APC and increase its immunogenicity. After treatment of Art v 1 with *cis*--aconitic and citraconic anhydrides, Art v 1 derivatives, caaArt v 1 and citArt v1, respectively, were obtained with 80 % of the amino groups modified. In the 2,3-dimethylmaleic anhydride-treated Art v 1, number of amino groups was similar to that in unmodified Art v 1 (Table I). As dimethylmaleyl groups easily hydrolyze at neutral pH,[21] it is supposed that Art v 1 was actually modified with 2,3-dimethylmaleic anhydride but that this derivative (dmaArt v 1) was hydrolyzed during the 20 h dialysis against PBS. All derivatives were completely soluble over a wide range of pH values (2.0–10).

TABLE I. Percent of remaining amino groups after Art v 1 treatment with citraconic, *cis*-aconitic and 2,3-dimethylmaleic anhydride, estimated by the TNBS method

Derivative	Amino groups, %
Art v 1	100±2.7
citArt v 1	23.1±1.8
caaArt v 1	22.2±1.5
dmaArt v 1	96.6±3.8

SDS PAGE demonstrated that caaArt v 1 and citArt v 1 were monomers with molar masses virtually indiscernible from that of unmodified Art v 1 (Fig. 1a). The size exclusion chromatograms (Fig. 2) show that, according to the retention times of the derivatives, citArt v 1 (t_r = 22.35 min) and caaArt v 1 (t_r = 21.83 min) had slightly increased molecular masses compared to unmodified Art v 1 (t_r = = 23.69 min). Retention of the monomeric structure, with molar masses similar to that of native Art v 1, as well as their complete solubility, makes these derivatives promising candidates as immunogens for allergen immunotherapy.

The native PAGE results show that the derivatives were very acidic in contrast to native Art v 1 (pI around 8), which did not even enter into the running gel (Fig 1b). caaArt v 1 was more acidic than cit Art v 1 because it has one carboxyl group more per introduced acyl group. By IEF, it was observed that the pI value of the derivatives was lower then 3.5 (results not shown). These results suggest that these derivatives with a very high negative charge density could be good SR ligands. Also with a so significantly altered structure, it is expected that the IgE binding would be dramatically reduced.

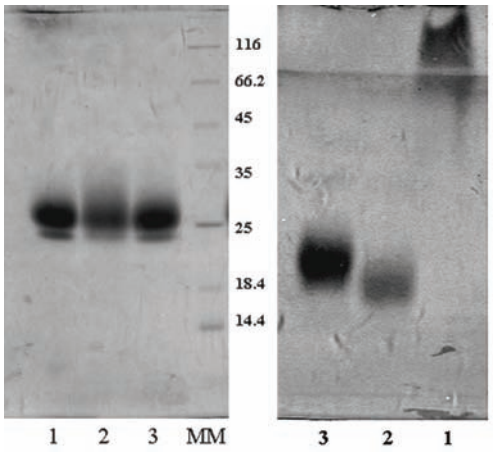


Fig. 1. a) SDS PAGE of unmodified Art v 1 (lane 1), caaArt v 1 (lane 2) and citArt v 1 (lane3); b) native PAGE.

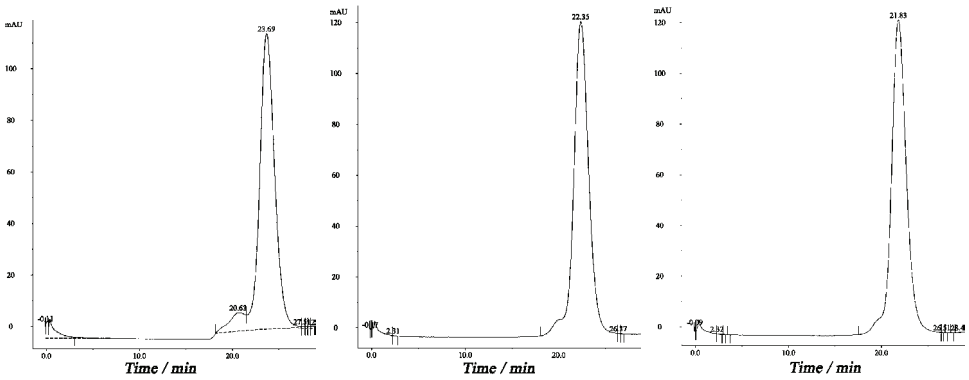
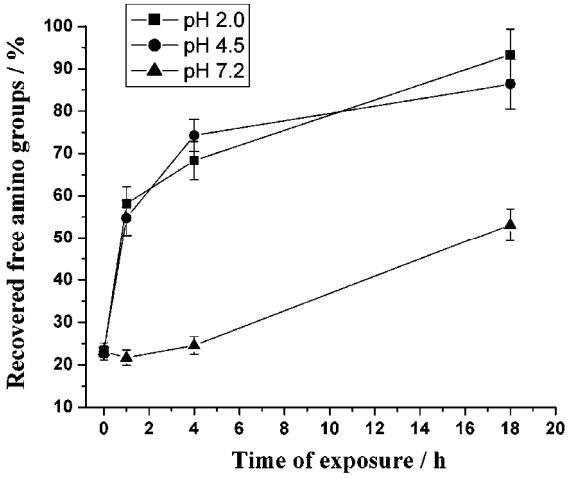


Fig. 2. Size exclusion chromatograms of native Art v 1 (panel A), citArt v 1 (panel B) and caaArt v 1 (panel C).

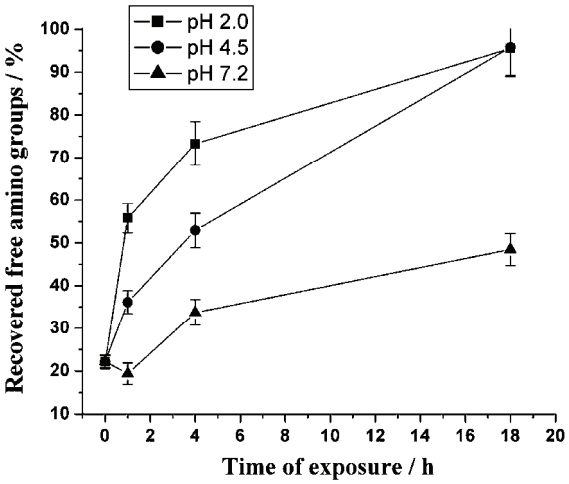
The pH stability test of the Art v 1 modifications showed that at physiological pH (pH 7.4), the half life is 30 and 25 h for caaArt v 1 and citArt v 1, respectively. At pH 4.5 (pH in the lysosomal compartment), the half-life of caaArt v 1 and citArt v 1 was 6 and 2 h, respectively. Finally, at pH 2.0, the half-life of both derivatives was about 2 h (Fig. 3). The stability of the derivatives at pH 7.0 should enable their relatively long half-life in circulation. On the other hand, their short half-life in an acidic environment, such as lysosomes during antigen processing, should enable these modified allergens to retain immunogenicity, *i.e.* to stimulate allergen specific T cells in a similar manner to native allergens.

In order for a protein to act as a good SR-ligand, it must have a certain negative charge density. On the other hand, a too high level of acetylation or succi-

nylation (which could provide this property) might decrease immunogenicity and T cell recognition. In contrast, a high degree citraconylation and *cis*-aconitylation generates two and three times greater negative charge density compared to acetylation (and, consequently, giving much better ligands for SR). A further potential advantage of the studied chemical modifications is the expected hydrolysis of acyl groups during antigen processing, which should allow the generation by an APC of the same set of peptides as the native allergen. This could be especially important because it was shown that T cell response to Art v 1 is characterized by one strong immunodominant epitope of 15 amino acids, containing up to three lysine residues.[22]



(a)



(b)

Fig. 3. pH stability of a) citArt v 1 and b) caaArt v 1. The results are presented as percent of the remaining amino groups of the modified Art v 1, taking the number of native Art v 1 amino groups as 100 %.

CONCLUSIONS

In this work, the preparation of reversibly modified Art v 1, the major mugwort allergen, by treatment with citraconic, *cis*-aconitic anhydride and 2,3-dimethylmaleic anhydridesis described. Acylation of Art v 1 by treatment with citraconic and *cis*-aconitic anhydride gave highly negatively charged derivatives (caaArt v 1 and citArt v 1). As 2,3-dimethylmaleyl derivative was hydrolyzed rapidly even at neutral pH values, this derivative was too unstable to be studied further and thereby was dismissed as a potential allergoid candidate. Additionally, the low stability of caaArt v 1 and citArt v 1 in an acidic environment would enable the complete retention of the specificity of the unmodified allergen. Modification of allergens by *cis*-aconitylation and citraconylation could be a new strategy for safer and more efficient allergen-specific immunotherapy. The derivatives obtained by citraconic and *cis*-aconitic anhydride treatment are suitable for further immunological testing.

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И З В О Д

ХЕМИЈСКЕ МОДИФИКАЦИЈЕ Art v 1, ГЛАВНОГ АЛЕРГЕНА *Artemisia vulgaris*, *cis*-АКОНИТИЛОВАЊЕМ И ЦИТРАКОНИЛОВАЊЕМ

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Art v1 је главни алерген полена црног пелина (*Artemisia vulgaris*), значајног узрочника поленске грознице широм Европе. Алерген-специфична имунотерапија је за сада једини делотворан начин за третирање алергија, при чему примена модификованих алергена чини овакав третман безбеднијим и ефикаснијим. У овом раду, два од три (анхидрид *cis*-аконитне, цитраконске и 2,3-диметилмалеинске киселине) испитивана анхидрида су се показала погодним за хемијске модификације алергена. Art v 1 је модификован *cis*-аконитиловањем и цитракониловањем у циљу добијања деривата Art v 1 погодних за даље имунолошке тестове. Ацеловањем Art v 1 добијени су деривати (caaArt v 1 и citArt v 1) са око 80 % измодификованих амино група. Добијени деривати су мономерни, са молекулском масом сличном нативном Art v 1, али са драматично смањеним pI вредностима. Оба деривата су релативно стабилна у неутралној, док се у киселој средини ацил групе хидролизују. Модификација алергена *cis*-аконитиловањем и цитракониловањем може бити нови начин за добијање алергоида.

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