

ORIGINAL PAPER

Allergenicity and immunogenicity of the major mugwort pollen allergen Art v 1 chemically modified by acetylation

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Clinical and Experimental Allergy

Summary

Background Treating allergies with modified allergens is an approach to make the treatment safer and more efficient. Art v 1 is the most prominent allergen of mugwort pollen and a significant cause of hayfever around Europe. The aim of this study was to reduce the allergenicity of Art v 1 by acetylation, and to investigate the capacity of the modified protein to generate blocking antibodies.

Methods The reduction of allergenicity of Art v 1 following acetylation was monitored by immunoblot, ELISA inhibition using a pool of sera from mugwort pollen allergic patients, basophil activation assay and by skin prick testing of mugwort-allergic patients. Rabbits were immunized against Art v 1 and acetylated Art v 1 (acArt v 1) and the rabbit antisera were tested for their capacity to block human IgE binding in ELISA. Human T cell proliferation against Art v 1 and acArt v 1 was examined in peripheral blood mononuclear cells (PBMCs) of mugwort pollen allergic patients and cytokine release in PBMC cultures was monitored.

Results Acetylation of Art v 1 gave a derivative of reduced allergenicity in the *in vitro* and *ex vivo* tests applied. The skin test reactivity to acArt v 1 was significantly reduced in 19 patients when compared with the reactivity to Art v 1. Rabbit antibodies to acArt v 1 and Art v 1 showed similar capacity to block human IgE binding to Art v 1 in inhibition ELISA. Both proteins were able to induce proliferation of PBMCs and CD3/CD4⁺ cells of mugwort-allergic patients. Release of IL-5 was significantly reduced in cultures stimulated with acArt v 1.

Conclusions Art v 1 modified by acetylation had a significantly reduced allergenicity *in vitro* and *in vivo*, while its immunogenicity was retained. Modification of allergens by acetylation could be a new strategy for allergen-specific immunotherapy.

Keywords acetylation, allergen-specific immunotherapy, allergoid, Art v 1, blocking antibodies, mugwort pollen allergy

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Introduction

Allergen vaccination is currently the only curative approach for treating allergic diseases. The mode of action involves modulation of antigen-presenting cell (APCs), T cell and B cell responses to the allergen accompanied by an increase in allergen-specific serum antibodies of the IgG class (so-called blocking antibodies) [1]. Its effective-

ness, especially in the treatment of allergic rhinitis, is well documented [2].

Even though it has been used for decades, allergen vaccination can be accompanied by severe side-effects and can sometimes be followed by anaphylactic reactions in a patient receiving treatment [3]. For these reasons, there is a demand for preparations of allergens that are effective in treating the disease but with reduced side-effects to be introduced into clinical practice and new formulations are still the subject of research. The aim is to

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modify allergens in order to reduce IgE binding, while preserving structural features necessary for T cell recognition and induction of allergen-specific IgG antibodies. Various forms of polymerized derivatives of allergens obtained by chemical modification with cross-linking agents [4, 5], monomeric allergoids (derivatives obtained by chemical modification of side-residues without causing polymerization of allergens) [6] and hypoallergenic preparations (derivatives of allergens obtained by recombinant technology) have been described as having those features and have proved promising in treating allergic diseases [7–10].

Simple, straight-forward methods leading to desirable properties of an allergen, e.g. reduction of allergenicity but with the potential to stimulate the immune system, are of great interest in order to provide new tools to improve allergy vaccination. Chemical modification of allergens may provide means to reduce allergenicity and introduce immunomodulatory properties into a protein by a single process. Acetylation, by blocking positively charged amino groups, renders treated proteins negatively charged. The molecular basis of recognition by a family of pattern-recognition receptors (PRRs), called scavenger receptors (SRs), expressed mainly on cells involved in early stages of the immune response to foreign antigens, has not been characterized in detail, but it is known that recognition requires negatively charged moieties on a ligand [11–13]. Similarly to toll-like receptors (TLRs), these receptors can bridge the innate and adaptive immunity and can induce adaptive immune responses to their ligands [14]. Many proteins are known to become SR ligands when chemically modified to enhance their negative charges with acetic or maleic anhydride, or physiologically with malondialdehyde [11, 15, 16]. As a result of enhanced uptake by APCs via SRs (and perhaps more efficient antigen processing), their immunogenicity is increased together with a dominantly Th1-type of immune response [17–19]. From the aspect of immunomodulation of an already established Th2 immune response, targeting SRs has potential in immunotherapy of allergies [17, 18].

Mugwort pollen is a frequent cause of allergic diseases in Europe. Its major allergen, Art v 1, a defensin-like glycoprotein with a characteristic hydroxyproline-rich tail [20] is frequently recognized by allergic patients [21, 22]. In this study we applied the acetic anhydride modification to purified Art v 1 and obtained a highly acidic derivative of Art v 1 which retained its monomeric structure. The modified protein exhibited reduced ability to bind IgE from the sera of allergic patients and to induce skin reactions in allergic persons. Rabbits immunized with acArt v 1 recognized the unmodified protein and the rabbit antibody produced inhibited the recognition of Art v 1 by IgE from mugwort pollen allergic patients.

Material and methods

Acetylation of Art v 1

Art v 1 was purified by high-performance liquid chromatography (HPLC) from mugwort pollen extract as described previously [23]. The protein was subjected to acetic anhydride treatment. Thus, Art v 1 (1 mg/mL) in 10 mL of water saturated with 1.36 g sodium acetate was exposed to acetic anhydride (5 μ L) in five aliquots for 30 min with extensive mixing. The mixture was dialyzed against water for 24 h. The modified protein was stored at -80°C . The molecular mass of the native and modified proteins was determined by size exclusion chromatography (SEC) on a Superdex 75 PC 3.2/3 column using an HPLC system Äkta Purifier 10 (Amersham Pharmacia, Uppsala, Sweden). The running buffer was 50 mM Tris (pH 8.2), 0.2 M NaCl and the flow was 50 μ L/min. Protein concentrations of native and modified proteins were determined by the UV spectroscopic method [24], using an extinction quotient of 0.665 calculated for Art v 1 as described previously [23].

Endotoxin determination

Endotoxin activity was determined in a limulus amebocyte lysate (LAL) endochrome assay (Charles River Endosafe, Charleston, SC, USA) according to the manufacturer's instructions. Endotoxin levels in Art v 1 and acArt v 1 were 6 and 8.8 ng/mg protein, respectively.

Amino group determination and acetylated Art v 1 stability in vitro

The number of amino groups in control and modified samples was determined using 2,4,6-trinitrobenzenesulphonic acid (TNBS) as a reagent according to a previously described procedure [25]. Results were expressed as means of three different determinations and for modified Art v 1 as a percentage of the number of amino groups determined for the unmodified sample (expressed as 100%). The stability of the derivative formed by acetylation was determined in a neutral environment [phosphate-buffered saline (PBS), pH 7.2] and in a slightly acidic solution (150 mM sodium acetate buffer, pH 4.5), by exposing the modified protein to these conditions for up to 6 h at 37°C . Aliquots for TNBS determination of amino groups were taken at 15, 30 min, 1, 2 and 6 h.

Human sera

Sera from 25 patients with a positive skin prick test (SPT) to mugwort pollen, a documented clinical history of mugwort pollen allergy and with no previous immunotherapy to mugwort pollen recorded were pooled for

immunoblotting and ELISA inhibition experiments. Sera from five persons negative to mugwort pollen in SPT and with IgE levels to the mugwort pollen below 0.35 kU/L were pooled and used as the negative control.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and isoelectrofocusing

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using a Hoefer scientific instrumentation apparatus (Pharmacia, Uppsala, Sweden) with a discontinuous buffer system. Native and modified samples (10 µg/well) were diluted fivefold in sample buffer (with or without 5% β-mercaptoethanol) and boiled for 5 min before the application on gels. Protein components were resolved on 12% polyacrylamide (PAA) gel with a standard protein mixture for molecular weight reference. Gels were stained with Coomassie brilliant blue R-250 to visualize the separated proteins. An electroblotter system (Serva, Heidelberg, Germany) was used for transfer to nitrocellulose membrane and checked by staining the proteins with 0.5% Ponceau S for 15 min.

Isoelectrofocusing was performed in 7% PAA slab gel in the presence of 2% Ampholytes (Pharmacia), pH 3.5–10, in a Multiphor electrophoretic system (Pharmacia) at 200 V for 2 h. Each sample was applied in 30 µg quantities per well. The pH gradient was determined by cutting the gel into strips and incubating in 10 mM KCl for 30 min. The proteins were subsequently transferred to a nitrocellulose membrane (0.45 µm pore size; Millipore, Billerica, MA, USA) by capillary force for 45 min.

Immunoblotting analysis

IgE binding proteins were probed by immunoblotting. The proteins were separated by SDS–PAGE, transferred to nitrocellulose as described above and detected by probing with sixfold diluted pooled human sera in a diluting buffer [30 mM Tris-buffered saline (TBS)–0.1% BSA]. As secondary antibodies alkaline phosphatase labelled monoclonal anti-human IgE (Sigma-Aldrich, Munich, Germany) were 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 (nitro blue tetrazolium; Sigma-Aldrich) in 10 mL of 100 mM Tris buffer, containing 150 mM NaCl and 5 mM MgCl₂ (pH 9.6).

Rabbit IgG binding was probed after SDS–PAGE or isoelectrofocusing and transfer to nitrocellulose in a similar way. The rabbit antibodies to Art v 1 or acArt v 1 were diluted 1 : 10 000 times in buffer and detected by anti-rabbit IgG antibodies labelled with alkaline phosphatase (Sigma-Aldrich) diluted 1 : 6000 times followed by

visualization of the IgG binding proteins as described above.

Inhibition of human immunoglobulin E binding in enzyme-linked immunosorbent assay by Art v 1 and acArt v 1

Briefly described, the microtitre plates (Nunc, Maxisorp, Roskilde, Denmark) were coated with 50 µL of Art v 1 (5 µg/mL) overnight at room temperature and washed with TBS–0.1% Tween 20 (TTBS). After blocking with 100 µL of 1% BSA in TTBS, 50 µL of twofold diluted human sera, incubated with 50 µL of serially tenfold diluted Art v 1 or acArt v 1 (concentrations ranging from 51.2 µg/mL to 0.033 ng/mL for Art v 1 and 162 µg/mL to 0.2 ng/mL for acArt v 1) in tubes for 30 min at 4 °C, were added to the plates. For ELISA inhibition experiments, a human serum pool was two times diluted in a diluting buffer containing TTBS–0.5% of BSA. For inhibition, serial dilutions of unmodified and modified samples were prepared in a buffer consisting of TBS–0.5% BSA. The remaining uninhibited IgE that binds to the Art v 1 coupled plate was detected using anti-human IgE antibodies labelled with alkaline phosphatase (Sigma-Aldrich) and 4-nitrophenyl phosphate (4-NPP) (Sigma-Aldrich) as the substrate. Absorbance was measured at 405 nm after adjusting the background with the negative control. The percentage of inhibition was calculated as follows: %inhibition = [OD of the test sample – (OD of the inhibited sample/OD of the test sample)] × 100. The test sample (positive control) was 50 µL of twofold diluted human serum incubated with 50 µL of diluting buffer.

Basophil activation assay

The expression of CD203c was performed as previously described [26]. Briefly, heparinized blood samples were taken from eight mugwort-allergic patients. Blood aliquots (100 µL) were incubated with dilutions of Art v 1 and acArt v 1 antigens (0.1, 1 and 10 µg/mL in eight patients and 0.01 µg/mL as an extra dilution in four patients), anti-IgE antibody as a positive control (1 µg/mL), or PBS as a negative control for 15 min (37 °C). After incubation, cells were washed with PBS and then incubated with 10 µL of phycoerythrin (PE)-labelled CD203c monoclonal antibody 97A6 (Beckman Coulter, Fullerton, CA, USA) and fluorescein-isothiocyanate (FITC)-labelled CD63 monoclonal antibody (Beckman Coulter) for 15 min at room temperature. Thereafter samples were subjected to erythrocyte lysis with 2 mL of FACSTM lysing solution (BD Biosciences, San Jose, CA, USA). Cells were then washed, resuspended in PBS, and analysed by flow cytometry using a FACSCalibur (BD Biosciences). The basophil marker CD203c was used to set a basophil gate including only CD203c⁺ cells. The collected cells were further analysed for granule release using an anti-CD63 antibody.

The magnitude of basophil activation was calculated as the percentage of CD63⁺ events among the gated basophils (CD63/CD203c).

Immunization of rabbits and determination of Ab levels by enzyme-linked immunosorbent assay

The antibodies against control (unmodified) and modified Art v 1 were raised in rabbits according to Harboe and Ingild [27]. Two rabbits were immunized with each protein and the serum obtained by pooling serum specimens of two rabbits were used for experiments. The presence of IgG antibodies was determined by ELISA using anti-rabbit IgG alkaline phosphatase labelled antibodies in a 1 : 6000 dilution (Sigma-Aldrich) and Art v 1 or acArt v 1 coupled to the microtitre plates (5 µg/mL). Rabbit serum was diluted 1 : 10 000 in a diluting buffer consisting of TTBS-0.5% BSA. Before the first immunization, serum samples were taken as the reference point, and then every 4 weeks after 51 days from the start of immunization.

Competition enzyme-linked immunosorbent assay for assessment of blocking antibody activity

The ability of rabbit anti-Art v 1 and anti-acArt v 1 to inhibit the binding of mugwort pollen-allergic patients' IgE antibodies to Art v 1 was examined by ELISA competition experiments. Instead of an inhibitor as in the inhibition ELISA described above, serial fourfold dilutions of rabbit antisera to Art v 1 or acArt v 1 were added to the plates for 30 min before the addition of twofold diluted pooled human sera. The dilutions were made in the diluting buffer described above. The percentage of blocking was calculated as follows: %blocking = [OD of the test sample - (OD of the blocked sample/OD of the test sample)] × 100. The test sample (positive control) was 50 µL of twofold diluted human serum incubated with 50 µL of buffer.

Lymphoproliferation assay in human peripheral blood mononuclear cell cultures

PBMCs were isolated from four mugwort-allergic patients by Ficoll (Amersham Biosciences) density gradient centrifugation. PBMCs (2×10^5) were cultured in triplicates in 96-well Nunclone plates in 200 µL of RPMI 1640 medium supplemented with 10% bovine growth serum (Hyclone, Logan, UT, USA), 2 mM glutamine (Sigma-Aldrich), 50 µM β-mercaptoethanol (Sigma-Aldrich), 25 µg/mL gentamicin, 100 IU/mL penicillin and 100 IU/mL streptomycin (Gibco, Invitrogen Corporation, Paisley, UK) at 37 °C and 5% CO₂ in a humidified atmosphere. Cells were stimulated with Art v 1, acArt v 1 (10 µg/mL) or unstimulated with medium alone in triplicate. Polymixin B (Sigma-Aldrich)

was added to the cultures at 1 µg/mL to minimize any effects of lypopolysaccharide on the antigen-specific proliferation in the cultures [28]. After 6 days, proliferative responses were measured by [³H] thymidine incorporation and expressed as stimulation indices (SI), i.e. counts per minute (cpm) value for stimulated cell cultures/cpm value for unstimulated cell cultures (mean of triplicates).

CD3/CD4⁺ cells proliferation assay in human peripheral blood mononuclear cells

To compare abilities with stimulate proliferation in PBMCs of Art v 1 and acArt v 1, we applied a modification of the method that was described for cell-mediated immune responses in whole blood after stimulation with viral antigens [29] and applied for cat allergens [26]. PBMCs obtained by Ficoll density gradient centrifugation of 10 mugwort-allergic patients were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 25 µg/mL gentamicin, 100 IU/mL penicillin and 100 IU/mL streptomycin (Sigma-Aldrich) and supplemented with 10% heat inactivated autologous plasma. Polymixin B (Sigma-Aldrich) was added to the cultures at 1 µg/mL. The PBMCs were stimulated with Art v 1, acArt v 1 (10 µg/mL) or unstimulated with medium alone in duplicates. Tetanus toxoid (TT) (5 µg/mL) was used as positive control (Torlak, Serbia). Cells were incubated in FACS tubes for 7 days at 37 °C and 5% CO₂ in a humidified atmosphere and stimulated as described above. After incubation, the cells were spun down for 5 min at 300 *g* at 4 °C and 100 µL of cell culture supernatants were withdrawn for cytokines analysis. Cells were washed with PBS and stained with αCD3-FITC and αCD4-PE (BD Pharmingen, San Diego, CA, USA) at 4 °C for 20 min and analysed using a FACSCalibur (BD Biosciences) with Cellquest software. As a measure of stimulation, the number of proliferating CD3/CD4⁺ cells (CD3/CD4⁺ cells among gated blasts) is expressed as the number of proliferating cells per 100 000 lymphocytes (resting and proliferating lymphocytes).

Supernatants collected for determination of cytokines (IL-4, IL-5, IL-10 and IFN-γ) were analysed using the Th1/Th2 cytometric bead array-kit (BD Biosciences) according to the manufacturer's instruction (detection limit 2.5 pg/mL). The supernatant specimens were stored at -80 °C until being analysed. Differences between the cytokine release to Art v 1 and acArt v 1 stimulation were compared statistically using the Wilcoxon matched pairs test and the Statistica 9.0 program.

Study population and skin prick testing

Nineteen subjects suffering from mugwort pollen allergy were included in the study (9 men and 10 women; aged 13–55 years, median 37 years). In addition, two subjects with a negative SPT to mugwort pollen extract were tested

with the acArt v 1. The samples for SPT were prepared at the Institute for Immunology and Virology, Torlak, Belgrade, and used at concentrations of 3 and 30 µg/mL. Weal diameters were expressed in relation to the histamine response using the same grading system as described elsewhere [30]. SPT was performed outside the mugwort pollen season, during the period from October to June. Differences between the skin test reactivity to Art v 1 and acArt v 1 were compared statistically using the Wilcoxon matched pairs test.

The study was approved by the Ethical Committee of the Zvezdara University Hospital, Belgrade, Serbia.

Results

Extent of Art v 1 modification and stability of the acyl bond at different pH values

The protein obtained by acetylation of amino groups in Art v 1 (acArt v 1) exhibited amino group modification of up to 80% when compared with non-modified Art v 1. We examined the stability of the bond formed in both neutral and acidic conditions. Incubation of acArt v 1 in phosphate buffer (pH 7.4) did not cause any deacetylation of the amino groups, while incubation of the protein for 6 h in slightly acidic conditions (pH 4.5) led to regeneration of some amino groups (15%).

Biochemical characterization of the modified Art v 1

acArt v 1 was completely soluble and did not form dimers or oligomers in native conditions, as demonstrated by gel filtration (not shown). Similarly, no aggregation was observed for acArt v 1 as monitored by turbidity of the solution before and after modification (results not shown). The retention times during SEC under the conditions described in 'Material and methods' were 23.8 min for Art v 1 and 22.8 min for acArt v 1. The molecular weights of native and modified Art v 1 were about 33 and 40 kDa, respectively. The extensive acetylation probably influenced the natural conformation of the protein, as the extent of modification determined by the TNBS method and the size of the newly introduced group cannot account for the shift in the molecular weight obtained by SEC.

The apparent size of the proteins differed in the SDS-PAGE. Thus, the mobility of the acetylated derivative was decreased in both non-reducing and reducing conditions when compared with Art v 1 (Fig. 1a), exhibiting an apparently higher molecular weight. The difference in apparent molecular size between the unmodified and the modified protein was more pronounced under reducing conditions. Abnormal mobility in the presence of SDS may occur with very acidic proteins, due to incomplete binding of SDS [31]. Staining with CBB dye was also weaker for acArt v 1 due to blocking of the amino groups responsible for binding [32, 33].

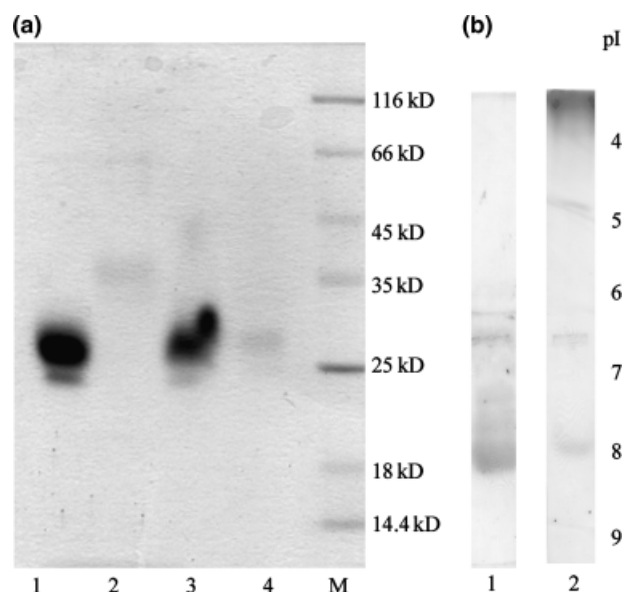


Fig. 1. Biochemical characterization of acetylated Art v 1. (a) SDS-PAGE of Art v 1 (lanes 1 and 3) and acArt v 1 (lanes 2 and 4), under reducing conditions (lanes 1 and 2) and non-reducing conditions (lanes 3 and 4). M: molecular weight marker proteins. Ten micrograms of protein were applied per well. (b) Isoelectric point determination of Art v 1 and acArt v 1 by isoelectrofocusing following transfer to the membrane and visualization by immunoblotting using Art v 1 and acArt v 1 rabbit antibodies. Thirty micrograms of protein were applied per well. Lane 1 – Art v 1, lane 2 – acArt v 1.

The pI value of acArt v 1 shifted towards a more acidic region of the gel during isoelectrofocusing, as the modification had blocked positively charged amino groups (Fig. 1b). Thereby the net charge of acArt v 1 can be considered negative under physiological conditions (isoelectric point around 3.8), whereas unmodified Art v 1 is positively charged and present in several isoforms (isoelectric point is around 8).

acArt v 1 exhibits reduced immunoglobulin E binding in vitro

Pooled sera from mugwort pollen allergic patients were used in immunoblotting and ELISA inhibition experiments. In both immunoblot (Fig. 2a) and ELISA inhibition (Fig. 2b), Art v 1 showed markedly greater binding of mugwort-specific IgE. The band corresponding to acArt v 1 could hardly be seen in the immunoblot assay, while comparison of the IC₅₀ values obtained in the ELISA inhibition (1.2 µg/mL for Art v 1 and 474.1 µg/mL for acArt v 1) showed that acArt v 1 inhibits binding of the allergic patients' IgE to the Art v 1 coupled microtitre plate nearly 400 times more weakly than the intact allergen.

When analysing individual patients regarding the ability of acArt v 1 to activate basophils (Fig. 3), some of the patients reacted to the acArt v 1 to a certain extent. However, the threshold concentration for basophil

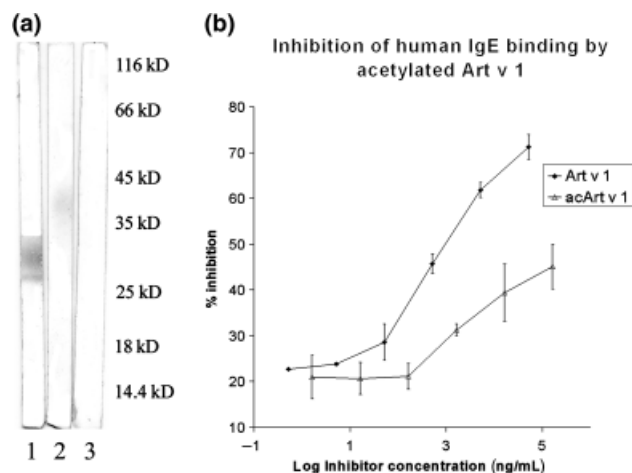


Fig. 2. IgE binding properties of acetylated Art v 1. (a) Immunoblotting detection of IgE binding in the pooled sera of mugwort pollen allergic patients. Lane 1 – Art v 1, lane 2 – acArt v 1, lane 3 – secondary antibody control, serum pool from non-atopic subjects incubated with Art v 1 blotted to the membrane. Ten micrograms of protein were applied per well. (b) ELISA inhibition of IgE binding to the Art v 1 coupled microtitre plate from the pool of mugwort pollen allergic patients' sera by Art v 1 and acetylated Art v 1.

activation was lower in the case of Art v 1 in a majority of patients tested.

acArt v 1 is immunogenic in animals and immunoglobulin G Abs induced by immunization with acArt v 1 inhibit patients' immunoglobulin E binding to the intact allergen

To test immunogenicity of the modified protein, animals were immunized and the IgG response generated was analysed by ELISA and immunoblot.

Rabbits immunized to acArt v 1 mounted an immune response that was equal in quality to the immune response to Art v 1 (Figs 4a and b). The rabbit IgG antibodies generated to acArt v 1 were able to recognize the unmodified protein in the immunoblot (Fig. 4c). Similarly, cross-reactivity was observed between the IgG antibodies generated to Art v 1 and acArt v 1, both in ELISA and immunoblot.

Our aim was to investigate the blocking potential of the antibodies generated to acArt v 1. In a blocking ELISA, Art v 1 was coupled to the plate and increasing amounts of rabbit sera generated to Art v 1, acArt v 1 or pooled pre-immune sera were added to the mugwort pollen allergic sera. The amount of IgE antibodies that remained unblocked and able to bind to the Art v 1 coupled plate was quantified in the assay (Fig. 5). The slopes of the blocking curves were similar for both proteins, while dilution of the rabbit antibodies used in the assay to block 50% of the human IgE differed fourfold between Art v 1 and acArt v 1. The pre-immune sera did not block the human IgE binding to the Art v 1 coupled microtitre plates when diluted eight times or more.

acArt v 1 is able to induce proliferation of peripheral blood mononuclear cells and CD3/CD4⁺ cells from mugwort pollen allergic patients

In order to test capacity of the modified protein to induce T cell proliferation, two assays were performed: proliferation in PBMCs monitored by thymidin-incorporation in four allergic patients and proliferation of CD3/CD4⁺ cells in PBMCs of 10 allergic donors. Both Art v 1 and acArt v 1 were able to induce proliferation of the PBMCs from three out of four mugwort pollen allergic donors (Table 1) at the concentration used in the assay. One patient (No. 4) did not respond with a positive stimulation index either to Art v 1 or acArt v 1.

The proliferation of specific CD3/CD4⁺ cells in PBMCs cultures of 10 mugwort-allergic donors was statistically significant compared with medium-stimulated cells regardless of the antigen used, Art v 1 ($P=0.049$) or acArt v 1 ($P=0.049$). The highest number of proliferating cells was detected in case of the positive control antigen (TT) ($P=0.007$). The results are shown in Fig. 6.

Cytokine response to Art v 1 and acArt v 1 stimulation in peripheral blood mononuclear cells cultures

In response to Art v 1 stimulation, increased amounts of IL-5 ($P=0.01$) and IL-10 ($P=0.005$) were detected in culture supernatants of PBMCs of 10 allergic donors (Fig. 7). The cytokine response to the acArt v 1 was characterized by a statistically significant increase in IL-10 ($P=0.009$) compared with unstimulated cultures. The cytokine profile in response to stimulation of PBMC exhibited clear differences between Art v 1 and acArt v 1 especially regarding IL-5 and IL-10 levels. The IL-5 secretion to acArt v 1 stimulation was completely abolished, yet cells responded to the acArt v 1 with IL-10 secretion that was higher than in the unstimulated cultures, but lower than in the cultures stimulated with Art v 1. The levels of IL-5 and IL-10 in response to acArt v 1 were significantly lower than the cytokine levels stimulated by Art v 1 ($P=0.017$ and $P=0.047$, respectively).

acArt v 1 showed reduced allergenicity in vivo

Allergic patients ($n=19$) reacted to acArt v 1 with a reduced weal size compared with Art v 1 at both tested concentrations (30 and 3 $\mu\text{g/mL}$). The results are shown in Table 2. Two control subjects showed no reaction to any of the proteins used in the SPT. Using the histamine grading system, the skin response was quantified and compared by the Wilcoxon matched pairs test. The difference was statistically significant ($P=0.033$) for the higher concentration used (30 $\mu\text{g/mL}$). The number of non-responders (patients that did not react in SPT with a weal size >3 mm) was higher when testing was performed with

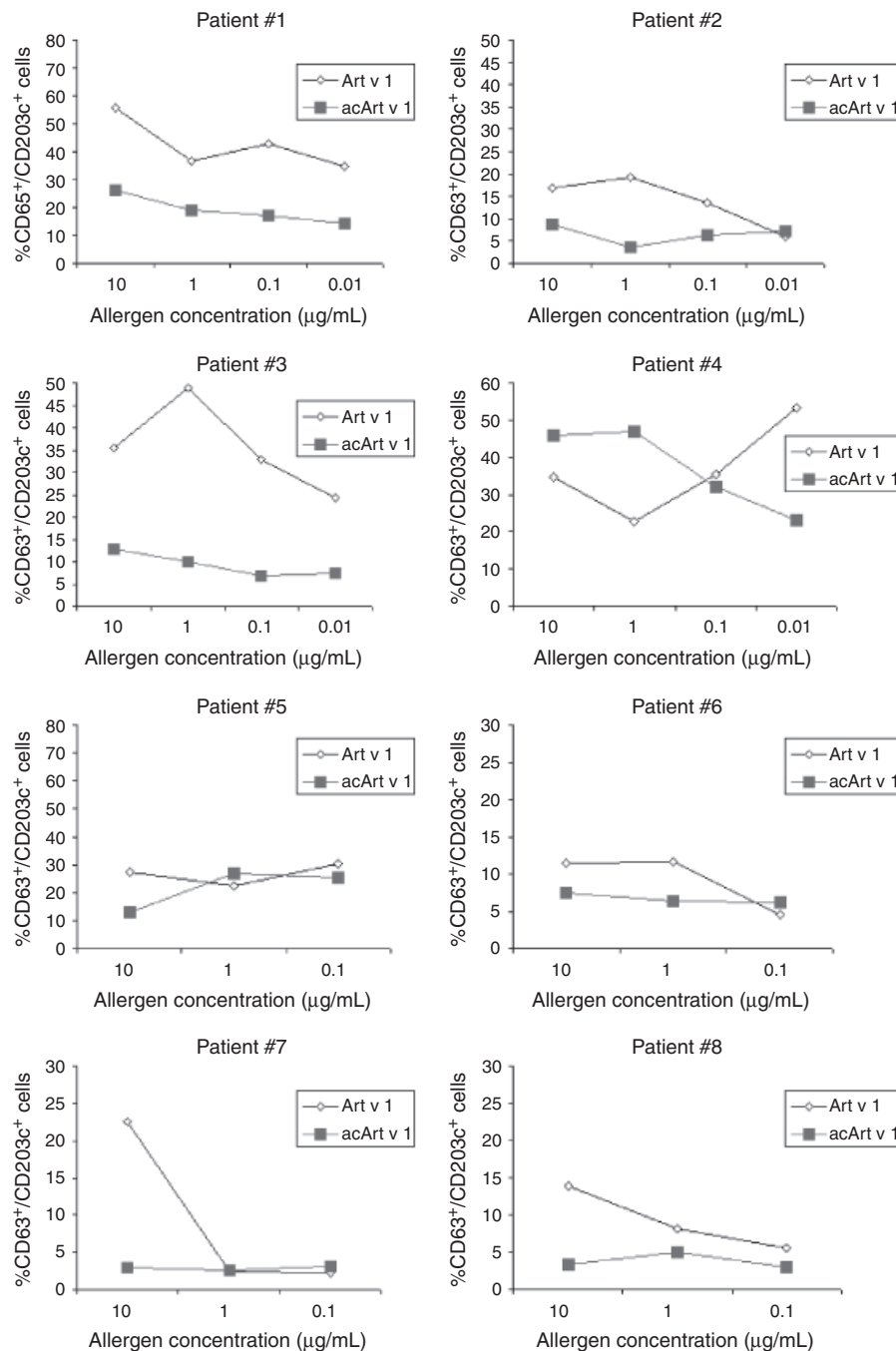


Fig. 3. Results of basophil activation assay in eight mugwort-allergic patients. The highest concentration used in the assay was 10 µg/mL for both antigens used.

acArt v 1 (17 for both tested concentrations) than with the Art v 1 (13 for both tested concentrations).

Discussion

Current and proposed new approaches to treat allergic diseases by allergen vaccination include the use of preparations made from intact allergenic extracts, those prepared by chemical modifications or those obtained by

recombinant technology. For some types of chemically modified allergens, retention of a monomeric structure makes them suitable for use in local allergen delivery routes. Monomeric allergens obtained by carbamylation have been described as safe and efficient in local immunotherapy trials both in adults and children [10, 34–36].

Here we prepared and characterized a monomeric allergoid of a major mugwort pollen allergen, Art v 1. We applied acetic anhydride treatment that renders positively

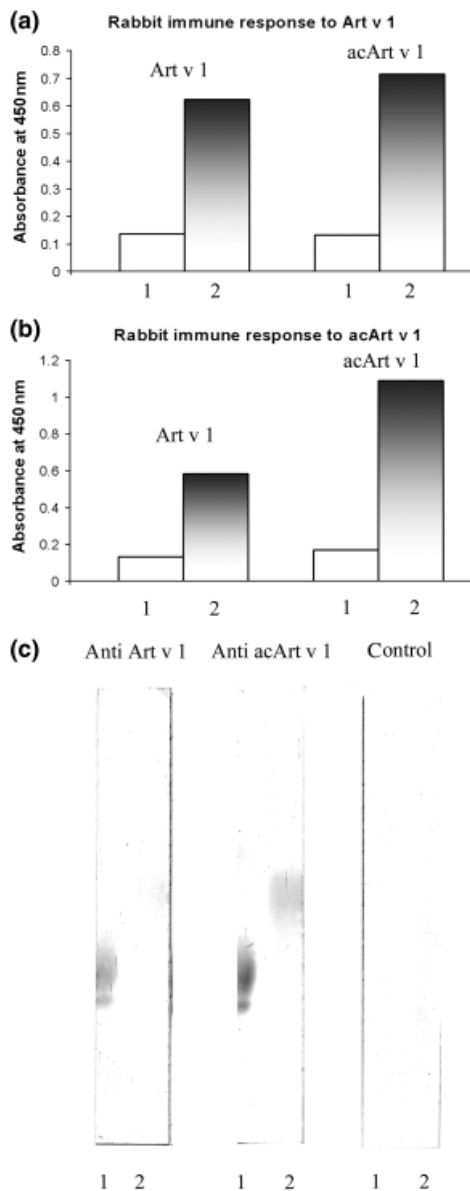


Fig. 4. Immunogenicity of acetylated Art v 1 demonstrated in rabbits. (a) Rabbit IgG titres in ELISA to the Art v 1 coupled microtitre plate. Two rabbits were immunized to Art v 1 (left columns) and acArt v 1 (right columns); mean values for two rabbits are depicted in the figure. Lane 1 – pre-immune sera, lane 2 – high titre immune sera. (b) Rabbit IgG titres in ELISA to the acArt v 1 coupled microtitre plate. Two rabbits were immunized to Art v 1 (left columns) and acArt v 1 (right columns); mean values obtained for two rabbits are depicted in the figure. Lane 1 – pre-immune sera, lane 2 – high titre immune sera. (c) Cross-reactivity of the obtained rabbit antibodies demonstrated in immunoblot. Lane 1 – Art v 1, lane 2 – acArt v 1. Membranes were probed with pooled sera obtained with Art v 1 or acArt v 1. The control membrane was probed with pooled pre-immune sera.

charged amino groups into acyl groups that are uncharged. The derivative formed is stable at neutral conditions, while a small amount of amino group unblocking occurs at acidic conditions. SEC confirmed that the monomeric nature of the obtained derivative was pre-

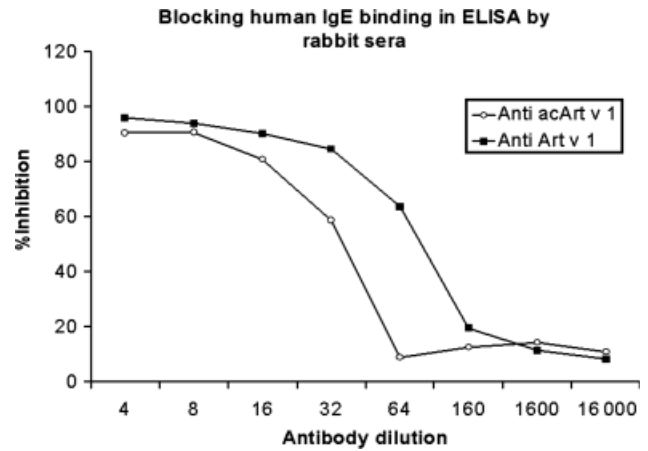


Fig. 5. Competition ELISA for assessment of blocking antibody activity. Art v 1 was coupled to the microtitre plate. The pooled sera of mugwort pollen allergic patients were pre-incubated with serial dilutions of rabbit antibodies generated to Art v 1 or acArt v 1. The remaining unblocked IgE that binds to the Art v 1 coupled to the plate, was detected using anti-human IgE labelled alkaline phosphatase antibody and 4-NPP as the substrate.

Table 1. Proliferation results in PBMC cultures of mugwort pollen allergic patients

Protein tested	Art v 1	acArt v 1
Patient #1	2.0	3.3
Patient #2	4.1	3.6
Patient #3	2.9	5.2
Patient #4	1.0	1.0

Proteins were tested at the concentration of 10 µg/mL. Stimulation indices were expressed relative to the medium only proliferation.

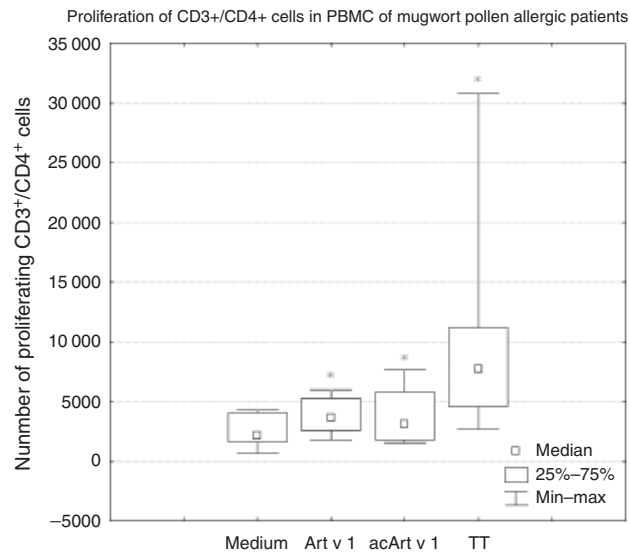


Fig. 6. Proliferation of CD3⁺/CD4⁺ cells in peripheral blood mononuclear cell (PBMC) cultures of 10 mugwort-allergic patients. Results are expressed as number of specific CD3⁺/CD4⁺ cells proliferating per 100 000 of total (resting and proliferating) lymphocytes. The statistical difference was calculated using Wilcoxon matched pairs test comparing stimulated cultures with unstimulated. $P < 0.05$ was considered significant (* $P < 0.05$).

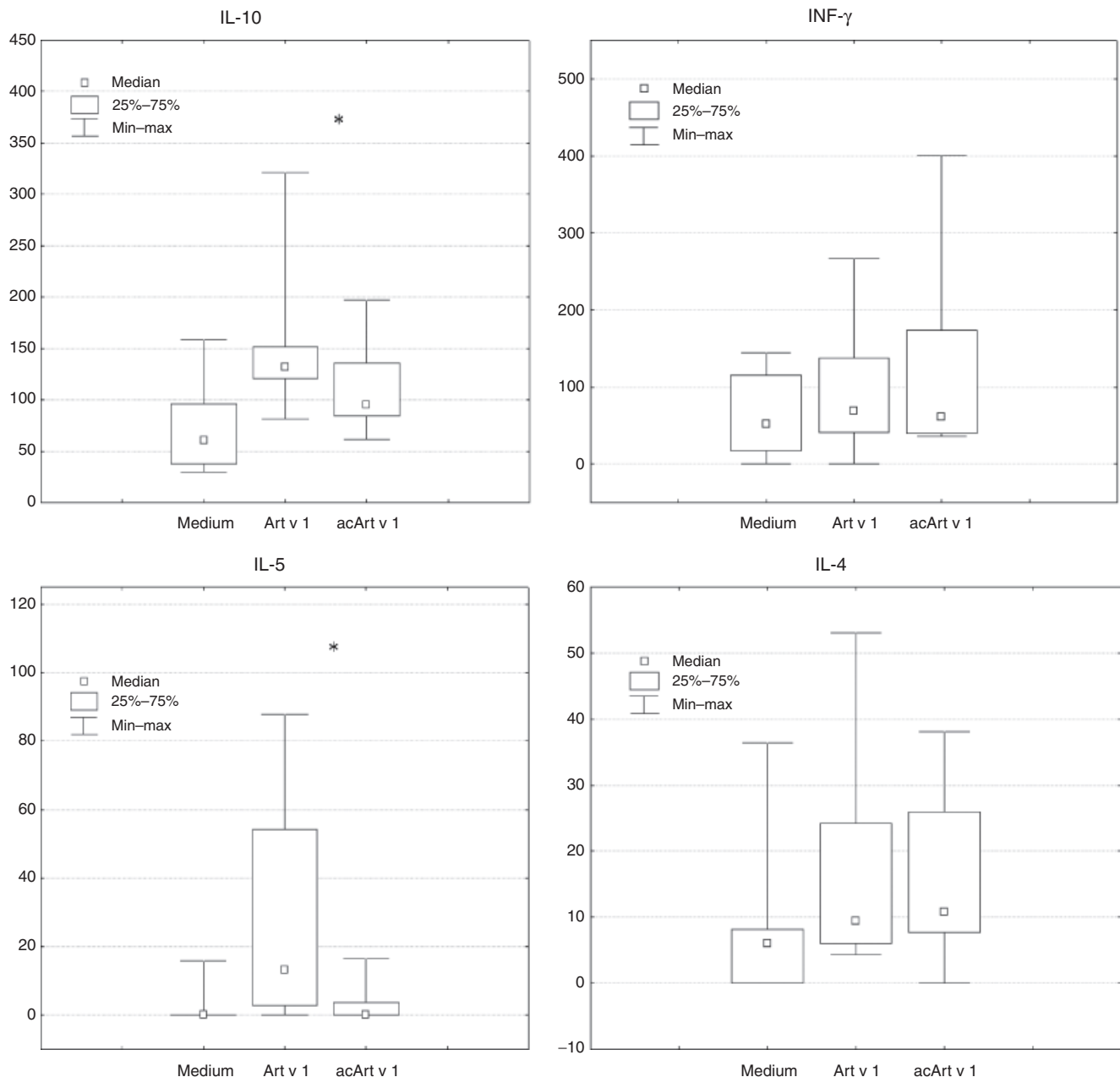


Fig. 7. Cytokine determination in 7-day cultures of peripheral blood mononuclear cell (PBMCs) stimulated with Art v 1 and acArt v 1. In response to Art v 1 stimulation, increased amounts of IL-5 ($P = 0.01$) and IL-10 ($P = 0.005$) were detected in culture supernatants. The cytokine response to the acArt v 1 was characterized by a statistically significant increase in IL-10 ($P = 0.009$) compared with unstimulated cultures. The levels of IL-5 and IL-10 in response to acArt v 1 were significantly lower than the cytokine levels stimulated by Art v 1 ($P = 0.017$ and $P = 0.047$, respectively). The statistical difference was calculated using Wilcoxon matched pairs test. $P < 0.05$ was considered significant (* $P < 0.05$). Statistical differences between Art v 1 and acArt v 1 are depicted in the figure (*).

served, when compared with the unmodified protein. All these features make this chemical approach suitable for making preparations for local delivery routes.

The derivatized protein, Art v 1, contains a positively charged defensin-like globe, which was targeted by the modification, and a tail, which should not be affected as it contains extensively glycosylated hydroxyproline repeats [20]. The low prevalence and affinity of IgE antibodies to

the glycocomponent of an allergen [37] can explain the marked reduction in human IgE binding in both *in vitro* assays applied here (ELISA and immunoblot). Chemical modification of the defensin-like part of the protein was sufficient to reduce the human IgE binding by almost 400 times in ELISA inhibition, and to decrease skin reactivity to the modified allergen. The weal size was reduced for the acetylated derivative compared with the unmodified

Table 2. Reactivity of Art v 1 and acArt v 1 in skin prick testing in 19 mugwort pollen allergic patients

Patient no.	Age/sex	Weal/flare						
		Saline	Histamine	Mugwort pollen extract	Art v 1 (30 µg/mL)	acArt v 1 (30 µg/mL)	Art v 1 (3 µg/mL)	acArt v 1 (3 µg/mL)
1	13/F	0/0	5/10	5/5	0/0	0/0	0/0	0/0
2	43/M	0/0	6/8	10/20	6/10	5/5	3/7	5/15
3	45/M	4/7	6/25	14/20	10/30	6/30	6/20	6/22
4	51/F	0/0	6/20	6/6	2/2	5/10	0/0	0/0
5	37/F	2/2	4/10	6/20	3/4	3/10	2/2	2/2
6	37/M	0/0	4/15	4/20	2/3	4/4	0/0	0/0
7	55/F	2/2	4/6	4/12	4/10	2/2	2/4	0/0
8	42/M	2/5	5/8	5/2	0/0	2/3	2/3	0/0
9	27/F	0/0	5/12	7/18	5/10	4/4	0/0	0/0
10	38/F	2/5	5/30	7/35	9/25	4/20	6/25	5/25
11	52/F	3/4	5/20	7/28	6/20	0/0	3/10	0/0
12	13/M	0/0	3/6	5/10	8/25	0/0	5/15	5/15
13	30/M	0/0	3/5	6/30	4/20	0/0	3/20	0/0
14	37/M	0/0	4/10	5/12	2/5	0/0	0/0	3/6
15	28/F	0/0	3/10	9/20	6/13	5/17	5/15	4/5
16	41/F	0/0	4/10	9/20	5/15	4/5	4/7	0/0
17	31/M	0/0	5/20	10/30	5/25	5/25	4/17	4/15
18	23/M	0/0	5/16	6/15	7/17	4/20	7/10	3/8
19	18/F	0/0	9/35	6/15	4/12	4/10	4/10	3/10

protein for both tested concentrations, the difference being significant for the higher allergen concentration. Lower sensitivity of an *in vivo* than an *in vitro* assay when comparing allergenic potencies of modified derivatives with their natural counterparts has been observed previously [30]. The existence of reactivity to acArt v 1 in some patients might be ascribed to the newly described IgE binding epitopes in this mugwort allergen residing solely in the hydroxyproline-linked arabinose moieties, which were not affected by the modification applied here [38]. Additionally, the very sensitive basophil assay applied to study ability of acArt v 1 to provoke basophil degranulation showed that especially with higher concentrations of acArt v 1 used in the assay, a population of very sensitive patients responded positively to the modified protein. It should be noted that there is a lower sensitivity of *in vitro* IgE binding assays, such as ELISA, compared with basophil activation assay.

Similar results of a weaker ability of commercially available allergoids (polymerized, glutaraldehyde treated) to evoke basophil activation were recently published [39], with the same observation on lower sensitivity of *in vitro* IgE binding assays, such as ELISA, compared with the basophil activation assay.

Another important property of a chemically modified allergen is its ability to evoke an immune response, i.e. its immunogenicity. Although allergenicity of the modified protein is reduced, yet not completely abolished, its immunogenicity was confirmed in both assays applied in this study: proliferation of PBMCs and CD3/CD4⁺ cells

proliferation in cultures stimulated for 7 days. The influence of minute amounts of LPS on allergen-specific proliferation was minimized by extensive antigen purification, monitored by LAL assay, and additionally controlled by the addition of polymyxin.

Art v 1 and its acetylated derivative displayed similar abilities to induce antigen-specific proliferation of PBMCs from three patients, and CD3/CD4⁺ cells of 10 mugwort-allergic donors. Cytokine response to the acArt v 1 was clearly different than to the Art v 1 and it was characterized by complete absence of IL-5 secretion to the modified allergen. An interesting observation is that the IL-4 levels remain unchanged by chemical modification of the stimulating allergen.

In addition, we showed that the immune response in rabbits to acArt v 1 was similar in quality and quantity to that for Art v 1. The obtained rabbit IgG antibodies to acArt v 1 cross-reacted with the unmodified protein and were able to block human IgE binding to Art v 1 coupled to the microtitre plate in a competition ELISA. The blocking potential was very similar to that obtained using antibodies to the unmodified protein. This is a very important feature as one of the outcomes of successful immunotherapy is the induction of allergen-specific IgG antibodies (blocking antibodies) [40].

By diminishing positive charges on the molecule, the remaining free carboxyl groups of aspartic and glutamic acid residues make the net charge of the modified protein very negative. The pI value of acArt v 1 in isoelectrofocusing was found to be <3.8 (compared with the

unmodified protein pI value of around 8). The specificity of SRs on macrophages and dendritic cells is not precisely known, but there are reports that they bind negatively charged ligands in a way that is not completely non-specific. Depending on the negative charge of the ligand and its distribution, different structures have different specificity for SRs, as was shown for lipoteichoic acid [41]. Similarly, binding of LPS by SR on Kupffer cells and endothelial liver cells depends on anionic groups in the lipid A moiety [42]. Binding to this family of PRRs has an immunomodulating effect on the Th response, skewing it towards Th1. More efficient antigen-processing and specific direction of allergen to macrophages could be a mode of action of chemically modified proteins designed to be potential SR ligands. This approach is suitable both for natural allergens and allergenic extracts, as it does not require a recombinant form of an allergen.

In conclusion, we obtained a chemically modified form of a major mugwort pollen allergen (Art v 1) exhibiting favourable features in all *in vitro* and *in vivo* tests applied for determination of the reduction of specific IgE binding and allergenicity. The protein mounted an immune response in animals and generated antibodies which cross-reacted with the unmodified protein and with blocking potential on human IgE binding. The highly negative charge of the obtained derivative suggests that this protein might be a possible ligand for SRs on macrophages, while retention of its native molecular weight could make it suitable for local administration forms of immunotherapy. However, the promising results obtained in the *in vitro* tests applied cannot be transferred immediately into clinical pathology.

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