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Concomitant sensitization to ragweed and mugwort pollen: who is who in clinical allergy?

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ABSTRACT

Background: In many areas of Europe, double sensitization to ragweed and mugwort is common, and because of the overlapping flowering periods of the 2 plants, it is not possible to diagnose the primary sensitizing allergen source and hence to determine the proper immunotherapy.
Objectives: To elucidate whether double-sensitized patients are cosensitized or cross-sensitized and, in the latter case, to define the primary sensitizer.
Methods: Serum samples from 34 patients with late summer respiratory allergy underwent skin prick testing with whole ragweed, and mugwort extracts were analyzed for their reactivity to recombinant Art v 1 and Amb a 1 by ImmunoCAP and then to Amb a 1, Art v 6, and Art v 1 isoforms by a proteomic approach. In double reactors, the primary sensitizing sources were detected by inhibition experiments.
Results: Serum samples from patients monosensitized to ragweed contained IgE to epitopes specific of all Amb a 1 isoforms. In contrast, serum samples from double reactors found to be primarily sensitized to mugwort reacted to Art v 1 and Art v 6 and cross-reacted to a few Amb a 1 isoforms. Finally, serum samples from double reactors found to be primarily sensitized to ragweed contained IgE reacting to all Amb a 1 isoforms, part of which cross-reacted to Art v 6. We did not find cosensitized patients.
Conclusion: This study found that Art v 6 plays an important role in mugwort allergy and that the cross-reactivity between Art v 6 and Amb a 1 is frequent, bidirectional, and clinically relevant in the area of Milan.

Introduction

An interesting phenomenon that is frequently observed by clinical allergologists working in areas where both ragweed and mugwort pollen are present is the unexpectedly high prevalence of concomitant sensitization to these pollens. In Lombardy, where the ragweed epidemic started in the 1980s, a marked increase in the prevalence of mugwort sensitization has been observed in parallel with the expansion of ragweed allergy. Notably, if individuals sensitized to pollen pan-allergens (profilin and polcalcins) are excluded, both sensitization and allergy to mugwort in the absence of ragweed hypersensitivity remain rare. At least 35% of ragweed-sensitized individuals living in the surroundings of Milan have a concomitant sensitization to mugwort on skin prick testing (SPT) with commercial pollen extracts.[1] In view of the overlapping flowering periods of these 2 Compositae plants, double sensitization represents a diagnostic dilemma for the clinical allergologists who have to decide whether to prescribe 1 or 2 distinct allergen-specific immunotherapies. These observations have prompted the consideration of potential cross-reactivity, in addition to shared profilin and procalcin sensitivity, between ragweed and mugwort pollen. More than 20 years ago, studies in the vicinity of Milan found that distinguishing between ragweed and mugwort allergy was virtually impossible.[2,3] In vitro analyses produced contrasting results that suggested the existence of cross-reactivity between allergens in the 2 pollen species (including the major mugwort pollen allergen, Art v 1) in some cases[4] and little or no cross-reactivity in other instances.[5,6] More recently, one study performed with serum samples from this area concluded that "patients showing both ragweed- and mugwort-positive SPT and/or RAST [radioallergosorbent test] are co-sensitized,"[1] despite the percentages obtained by skin tests, which strongly suggested a cross-reactivity (93% of mugwort-sensitized patients had ragweed sensitization and 38% of ragweed-allergic patients had mugwort sensitization).[1]

In recent years, the availability of natural purified or recombinant allergen proteins for diagnostic purposes has profoundly

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influenced our understanding of allergic phenomena. For instance, in patients living in northern Italy, the use of these new powerful tools has revealed that the serum samples from 47 of 105 consecutive ragweed-allergic patients (45%) had IgE reactivity to Art v 1 as well (Asero R 2012, unpublished results). Because Art v 1 has been considered as a marker of genuine sensitization to mugwort, this seems to confirm that patients sensitized to both weeds on skin tests are in effect cosensitized[1] and should be prescribed 2 distinct allergen extracts for immunotherapy. However, things are seemingly not that simple. Leonard et al[7] found that a minor ragweed allergen, Amb a 4, is homologous to Art v 1 and demonstrated a high degree of cross-reactivity between these 2 proteins by inhibition experiments. Furthermore, they observed that many more Austrians (who are frequently primarily allergic to mugwort) than northern Italians (who are frequently primarily sensitized to ragweed) react to Amb a 4 and that 42% of Art v 1–sensitized patients react to Amb a 4.[7] Notably, in that study Amb a 4 was recognized by approximately 30% of serum samples from ragweed-allergic patients, a proportion that is similar to the percentage of ragweed-allergic patients with cosensitization to mugwort.[1] Furthermore, another pair of cross-reacting allergens, Amb a 1 and Art v 6, subsequently have been detected in ragweed and mugwort pollen, respectively.[8] Although both allergens seem able to act as primary sensitizers, it was found that Amb 1 possesses more IgE epitopes than Art v 6 and, hence, dominates the cross-reactivity with its mugwort counterpart.[8]

The clinical significance of all these findings can be summarized by stating that the detection of reactivity to mugwort (and even to Art v 1) in the presence of ragweed allergy may not indicate primary mugwort sensitivity. On the other hand, the same may hold true if Amb a 1 hypersensitivity is found. Thus, despite the conclusions of the former study,[1] we believe that the issue of cross-reactivity in patients with double sensitization to mugwort and ragweed deserves to be reexamined with the new proteomic techniques.

Methods

Patients

Serum samples for this study were collected from adults with a history of seasonal, summertime (middle of August to the end of September) respiratory symptoms (rhinoconjunctivitis with or without asthma) who were addressed by their family physician to the allergy outpatient department of the Clinica San Carlo (Paderno Dugnano, Italy) and the allergy outpatient department of the Pordenone Hospital for allergy evaluation. All patients underwent SPT with commercial extracts (Allergopharma, Reinbeck, Germany) of the main seasonal airborne allergens present in Italy, including ragweed, mugwort, grass, pellitory, plantain, birch, olive, and cypress, and scored positive on SPT with ragweed extract. All clinical investigations were performed according to the principles of the Declaration of Helsinki; all patients gave their written informed consent to diagnostic procedures. The study was based on data stemming from routine clinical activity and on stored serum samples previously used to perform routine clinical investigations; the study has been approved by the institutional review board.

To avoid the interference by cross-reacting plant pan-allergens, such as profilin or calcium-binding proteins,[9] only patients sensitized to fewer than 3 pollens other that mugwort and/or ragweed were finally included. Furthermore, profilin hypersensitivity was ruled out by negative commercial profilin SPT results (ALK-Abellò, Madrid Spain). Serum samples from all patients underwent the measurement of IgE to recombinant (r) Amb a 1, the major ragweed allergen, and those from patients with positive SPT results with mugwort pollen extract were screened for their reactivity to rArt v 1, the major mugwort allergen. IgE were measured by the ImmunoCAP assay (Thermo Fisher–Phadia, Uppsala, Sweden); levels greater than 0.35 kU/L were considered positive results. Serum samples selected for immunochemical analysis were diluted 1:10 in Tris-buffered saline with Tween 20 (TBS-T) (20-mmol/L Tris, 150-mmol/L sodium chloride, and 0.05% [vol/vol] Tween 20, pH 7.5) and stored at 20°C until use.

Preparation of Pollen Protein Extracts

Soluble protein extracts of *Ambrosia artemisiifolia* L and *Artemisia vulgaris* L pollen were prepared according to Aina et al[10] by suspending 0.1 g of commercial pollen (Allergon, Ängelholm, Sweden) in 1 mL of bidistilled sterile water that contained protease inhibitor (1-mmol/L phenylmethylsulfonyl fluoride). Samples were incubated on a rotating drum for 2 hours at room temperature. The soluble fraction was isolated by means of 2 centrifugations at 13,000 × *g* for 10 minutes at 4°C and then stored at −20°C until use.

For 2-dimensional electrophoresis analysis, samples were purified with a clean-up kit (Bio-Rad Laboratories, Hercules, California) and dissolved in isoelectric focusing (IEF) rehydration buffer (7-mol/L urea, 2-mol/L thiourea, 2% [wt/vol] CHAPS (3-[(3-Cholamidopropyl)Dimethylammonio]-1-Propanesulfonate)), 20-mmol/L Tris hydrochloride, pH 8.8, 20-mmol/L dithiothreitol (DTT), 0.5% ampholyte mixture carrier, pH 3–10, 0.005% bromophenol blue). Protein concentration was assayed according to Bradford[11] using bovine serum albumin as standard.

Two-dimensional Electrophoresis and Immunoblotting

IEF was performed on a 7-cm immobilized pH gradient strips (Bio-Rad), providing a linear pH 4 to 7 gradient (for ragweed extract) or a nonlinear pH 3 to 10 gradient (for mugwort extract). Strips were rehydrated in 200 μL of rehydration buffer (7-mol/L urea, 2-mol/L thiourea, 2% [wt/vol] CHAPS, 20-mol/L DTT, 0.5% ampholyte mixture carrier, pH 3-10, 0.005% bromophenol blue) that contained 100 μg of protein sample. Passive rehydration (up to 10 hours) and IEF were performed at 20°C using a Protean IEF-Cell (Bio-Rad Laboratories).

After the first dimension separation, the IPG strips were equilibrated for 15 minutes against 6-mol/L urea, 30% glycerol, 2% sodium dodecyl sulfate, 0.375 M Tris hydrochloride, pH 8.8, and 2% DTT to resolubilize proteins and reduce disulfur bonds. The sulfhydryl groups were then blocked by substituting the DTT with 2.5% iodoacetamide in the equilibration buffer for 15 minutes.

After equilibration, strips were placed on the top of vertical 10 × 9-cm × 1.5-mm polyacrylamide gels (14% vol/vol). An agarose solution (0.5% low melting agarose in running buffer) was loaded to the top of the gel to lock strips, and electrophoresis was performed at 4°C in a Laemmle running buffer (25-mmol/L Tris hydrochloride, pH 8.3, 192-mmol/L glycine, 0.1% sodium dodecyl sulfate).[12]

Gels were run in the electrophoresis chamber (Mini-Protean electrophoresis system, Bio-Rad Laboratories) in parallel and used for protein revealing or immunoblotting experiments. For protein detection, gels were stained with colloidal Coomassie Blue G250 (0.1% Coomassie Blue G250, 170 g/L of ammonium sulfate, 34% methanol, and 3% phosphoric acid). For immunodetection experiments, gels were electroblotted (100 mA, overnight at 4°C) onto nitrocellulose membranes (0.45 μm, Bio-Rad Laboratories) by a Trans-Blot cell apparatus (Bio-Rad Laboratories) that contained transfer buffer (25-mmol/L Tris, 192-mmol/L glycine, and 20% [vol/vol] methanol, pH 8.3). Nitrocellulose membranes were then blocked with 5% (wt/vol) nonfat dry milk powder in TBS-T (20-mmol/L Tris, 150-mmol/L sodium chloride, and 0.1% [vol/vol] Tween 20, pH 7.5) for 1 hour, rinsed in TBS-T 0.05%, and incubated with 1:10 diluted serum. Bound IgE was detected with a horseradish peroxidase–conjugated goat anti-human IgE antibody (1:15,000 dilution, Sigma-Aldrich, St Louis, Missouri) followed by ECL assay with a commercial kit (Immun-Star Western C Kit, Bio-Rad Laboratories).

Table 1
Summary of the experimental results

Group	Skin test		Recombinant Art v 1 by ImmunoCAP	Immunoblot IgE reactivity			Primary sensitizer
	Amb	Art		Natural Amb a 1	nArt v 1	nArt v 6	
1	+	−	−	High reactivity to all the isoforms	−	−	Ragweed
2	+	+	+	Predominant reactivity with 1.01 isoform	+	+	Mugwort
3	+	+	−	High reactivity to all the isoforms	−	+	Ragweed
4	−	+	+	No or negligible reactivity	+	+	Mugwort

Spots were visualized on an x-ray film (Kodak, Rochester, New York). Serum samples from 25 patients (8 Amb+/Art+, rArt v 1+; 7 Amb+/Art+, rArt v 1−; and 10 Amb+/Art−) were tested.

LC-MS/MS and IgE-Binding Proteins Identification

Immunoreactive bands were carefully excised from Coomassie-stained 2-dimensional gels and submitted to in-gel trypsin digestion according to Aina et al[10] with minor modifications. The tryptic fragments were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). For the experiments, a linear ion trap (LTQ Orbitrap XL; Thermo Fisher Scientific, Waltham, Massachusetts) high-resolution spectrometer, equipped with a reverse-phase, high-performance liquid chromatography system, was used.

Protein identity was searched after peptide sequence attributions with Global Proteome Machine software (http://www.thegpm.org) with X!Tandem algorithm. The data used was created from downloads of 67 protein sequences from UniProt website (http://www.uniprot.org) using the words *pollen allergen Amb* as query.

All peptides were analyzed using Basic Local Alignment Tool Mass Spectrometry software (http://dove.embl-heidelberg.de/Blast2/msblast.html) to obtain the putative protein identity. Peptide sequences that were not identified through this database search method were further analyzed for a de novo peptide sequencing with Peaks Software (http://www.bioinfor.com).[13]

Immunoblot-Inhibition Assay

Immunoblot-inhibition experiments were performed according to Asero et al.[1] Each 1:10 TBS-T diluted serum sample was preincubated overnight (4°C) with increasing amounts (ranging from 2.5 to 10 μg of total proteins per 500 μL of diluted serum) of ragweed or mugwort pollen extract. IgE reactivity was detected before and after the absorption of serum with the extract following the protocol described above.

Results

Patients, rAmb a 1 IgE Levels, and Reactivity to Amb a 1 Isoforms

A total of 34 patients were studied. Thirty-two (all from the area of Milan) had strong IgE reactivity to rAmb a 1, with levels ranging from 3.23 kU/L to greater than 100 kU/L (reference range, <0.35 kU/L). On the basis of SPT with ragweed and mugwort extracts and on ImmunoCAP assay results, patients were grouped as follows (Table 1): (1) patients sensitized to ragweed but not to mugwort pollen on SPT (Amb+/Art−; n=10); (2) patients sensitized to both mugwort and ragweed pollen in vivo (Amb+/Art+), showing circulating IgE to Art v 1 on ImmunoCAP assay (n= 15) (in these patients, rArt v 1 IgE levels ranged from 1.09 kU/L to 23.7 kU/L); (3) patients sensitized to both mugwort and ragweed pollen in vivo (Amb+/Art+), scoring negative to Art v 1 on ImmunoCAP (n= 7); and (4) patients sensitized to mugwort but not to ragweed pollen on SPT (Amb−/Art+; n=2, both from Pordenone, an area where ragweed is virtually absent).

The serum from each patient was tested for its reactivity to the different Amb a 1 isoforms through a 2-dimensional immunoblotting analysis. Before this analysis, a reference 2-dimensional map of Amb a 1 isoforms was developed to define the allergen isoforms immunoreacting with the single serum sample. Figure 1 shows the 2-dimensional electrophoresis map of *Ambrosia artemisiifolia* pollen proteins from which 6 of the most significant spots recognized by a pool of serum from patients with ragweed allergy (Fig 1B) were excised and analyzed by LC-MS/MS. Immunoreactive spots 1 and 3 were attributable to Amb a 1.01 isoforms. Spot 2 was also mainly referable to an Amb a 1.01 isoform, although negligible amounts of Amb a 1.02 and Amb a 1.04 were also detected. This finding indicates a high similarity among these isoforms and a low expression of Amb a 1.02 and Amb a 1.04 compared with Amb a 1.01 in mature ragweed pollen. In contrast, spots 4, 5, and 6 were all referable to Amb a 1.03 isoforms.

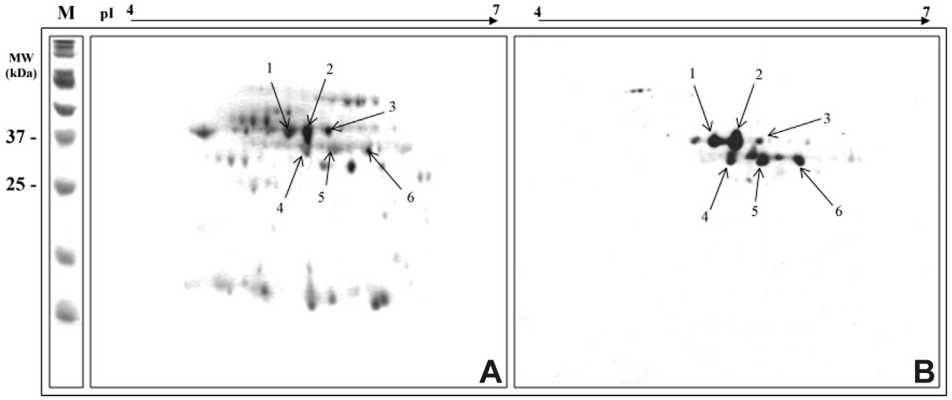

Figure 1. Two-dimensional protein and allergen maps of ragweed pollen. A representative 2-dimensional protein map of *Ambrosia artemisiifolia* pollen stained with colloidal Coomassie G-250 *(A)* and the related 2-dimensional immunoblotting map obtained by using a pool of serum samples from patients allergic to ragweed *(B)* is shown. *Arrows* show the 6 spots excised from polyacrylamide gel that after liquid chromatography tandem mass spectrometry analysis were referable to the following Amb a 1 isoforms: spot 1: Amb a 1.01; spot 2: mainly Amb a 1.01 but also 1.02, 1.04; spot 3: Amb a 1.01; and spots 4, 5, and 6: Amb a 1.03.

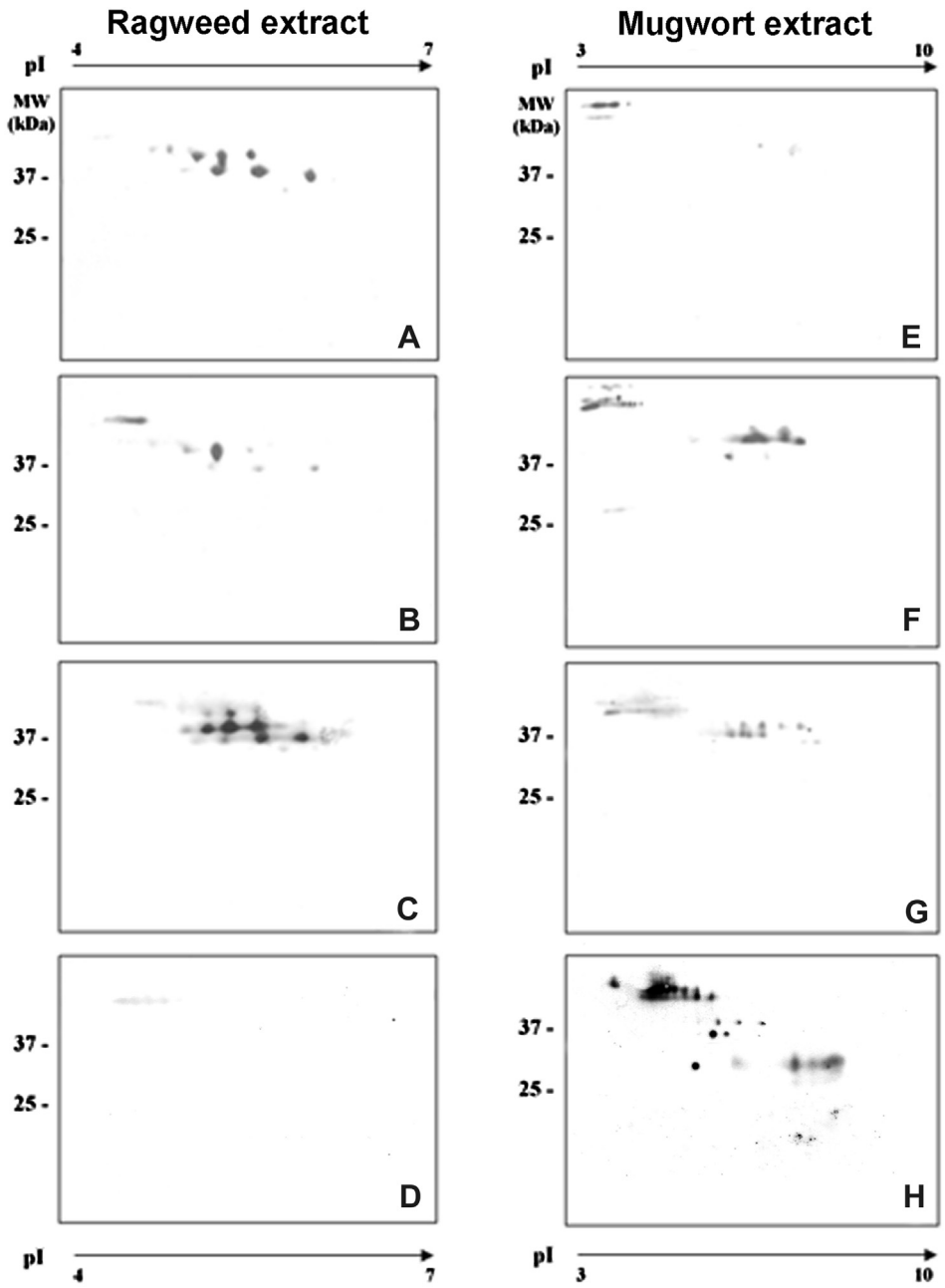

Figure 2. Serum reactivity to ragweed and mugwort proteins. Representative 2-dimensional immunoblots showing the IgE reactivity of the following 4 groups of patients' serum samples with ragweed *(A-D)* and mugwort *(E-H)* soluble pollen proteins: group 1 *(A, E)*: patients sensitized to ragweed but not to mugwort pollen (Amb+/Art−); group 2 *(B, F)*: patients sensitized to both mugwort and ragweed pollen (Amb+/Art+), showing circulating IgE to Art v 1 on ImmunoCAP assay; group 3 *(C, G)*: patients sensitized to both mugwort and ragweed pollen (Amb+/Art+), scoring negative to Art v 1 on ImmunoCAP; group 4 *(D, H)*: patients sensitized to mugwort but not to ragweed pollen (Amb−/Art+). Group 2 has a much more limited IgE reactivity to Amb a 1 isoforms than group 3.

Once the reference map was achieved, the differences in IgE reactivity to Amb a 1 isoforms of the 34 single serum samples were evaluated. The maps obtained for patients sensitized only to ragweed (Amb+/Art−) showed all 6 immunoreactive spots characterized in the reference map in all cases. A representative map obtained for this first group of patients is reported in Figure 2A. Notably, the signal intensity was very high and similar among the spots, suggesting that all these patients were sensitized to all the main Amb a 1 isoforms expressed in ragweed pollen. In contrast, serum samples from all the patients sensitized to both ragweed and mugwort and positive to rArt v 1 on ImmunoCAP (Amb+/Art+, rArt v 1+) generally had a less intense reaction with Amb a 1 isoforms except for the isoforms present in spot 2 (ie, mainly Amb a 1.01; Fig 2B). The maps obtained for all the serum samples from the third group of patients, also sensitized to both ragweed and mugwort but negative for rArt v 1 on ImmunoCAP (Amb+/Art+, rArt v 1−), were similar and indistinguishable from the maps of the first group of patients (Amb+/Art−, Fig 2C). Finally, no Amb a 1 isoforms were bound by IgE from serum samples of patients sensitized only to mugwort (Amb−/Art+, Fig 2D).

To better understand the meaning of these differences in reactions to Amb a 1 isoforms and their possible relationship with mugwort allergens, the reactivity of the same serum samples against mugwort proteins was evaluated. Figure 2E–H, show

representative 2-dimensional immunoblots. In keeping with SPT results, no mugwort specific allergens were recognized by Amb+/Art− patients' serum samples (Fig 2E). On the contrary, at least 2 isoforms of Art v 6 were recognized by all the serum samples from the other groups of patients (Fig 2F, G, and H), and some isoforms of Art v 1 were additionally recognized by serum samples from both Amb−/Art+ and Amb+/Art+, rArt v 1+ patients (Fig 2F and H). The intensity of immunoreactive signals related to Art v 6 proteins was often higher than those related to Art v 1 allergen, probably because of a negative effect of reduction and alkylation processes, occurring during 2-dimensional analysis, on IgE reactivity to Art v 1.[14] However, the higher reactivity of Art v 6 compared with that of Art v 1 was also a characteristic of some serum samples investigated in previous works.[1,15]

Immunoblot-Inhibition Assay

To determine whether mugwort and ragweed reactivity in the 2 groups of patients sensitized to both pollens (Amb+/Art+, rArt v 1+ and Amb+/Art+, rArt v 1−) was the result of a cosensitization or corecognition, 2-dimensional immunoblot inhibition experiments were performed using mugwort and ragweed pollen extracts as inhibitors. In Amb+/Art+, rArt v 1+ patients, preincubation of serum samples with mugwort extract led to a complete inhibition of IgE binding to all Amb a 1 isoforms (Fig 3A and B), whereas preincubation with ragweed extract caused only a partial reduction in Art v 6 reactivity (Fig 3C and D), suggesting that primary source of sensitization in all Amb+/Art+, rArt v 1+ patients examined was mugwort. In Amb+/Art+, rArt v 1− patients, preincubation of serum samples with mugwort pollen extract caused little or no reduction in Amb a 1 signal (Fig 3E and F), whereas preincubation of serum samples with ragweed pollen extract caused the complete inhibition of IgE binding to Art v 6 isoforms (Fig 3G and H), suggesting that for this group of patients the primary sensitizer was ragweed. The same results were obtained by 1-dimensional immunoblotting (data not shown). The overall results are summarized in Table 1.

Discussion

The present study was started with a practical aim: to define whether patients with seasonal symptoms of airborne allergy in August and September and a double sensitization to ragweed and mugwort pollen on SPT should undergo immunotherapy with 1 or 2 distinct pollen extracts. This problem was already addressed in 1 previous study,[1] which found a probable cosensitization to ragweed and mugwort. However, not all ragweed and mugwort allergens were investigated on that occasion, and the recent information coming from the work by Jahn-Schmid et al[8] reporting a high degree of cross-reactivity between the major ragweed pollen allergen Amb a 1 and its homologous mugwort allergen Art v 6, along with the ongoing observation that in the area of Milan mugwort hypersensitivity is virtually found only in ragweed-hypersensitive individuals, prompted us to perform this study.

On the basis of both skin tests with pollen extracts and the presence of IgE to rArt v 1, we divided patients into 4 subsets: Amb+/Art−; Amb +/Art, + rArt v 1+; Amb+/Art+, rArt v 1−; and Amb-/Art+. Patients belonging to the first subgroup were unquestionably monosensitized to ragweed and recognized all isoforms of Amb a 1; these patients did not recognize any allergen in mugwort pollen, indicating that they produced a multitude of diverse IgE antibodies against epitopes specific of the various Amb a 1 isoforms. In contrast, all the patients in the second subgroup (Amb+/Art+, rArt v 1+) recognized not only Art v 1 in mugwort pollen but also Art v 6, some epitopes of which were mugwort specific, whereas others were shared by Amb a 1, particularly by the isoform Amb a 1.01. On the basis of the results of our inhibition experiments, these individuals appeared to be primarily sensitized to mugwort, and their reactivity against ragweed extract on SPT and to rAmb a 1 in vitro appeared as the result of the cross-reactivity between Art v 6 and Amb a 1, the former being the primary sensitizer. This finding is in agreement with the results of a previous study that found that Art v 6 can act as a primary sensitizer and contains epitopes which cross-react with some Amb a 1 epitopes.[8] The same study also found that Art v 6 cross-reactive epitopes are few. This information helps to explain the 2-dimensional Amb a 1 isoform pattern typically produced by Amb+/Art +, rArt v 1+ patients' serum samples, which was characterized by a signal restricted to only one or a few Amb a 1 isoforms; this pattern was probably the result of a primary sensitization to mugwort and only subsequent corecognition of few isoforms of Amb a 1 by a small fraction of IgE that were originally directed to Art v 6. Thus, we can suppose that patients with IgE reactivity to Amb a 1, Art v 6, and Art v 1 are first sensitized to mugwort and only subsequently to ragweed through a cross-reactive mechanism. This finding is in keeping with the results of a Swiss epidemiologic study that found that, in that area, the observed sensitization to Ambrosia is in most cases a consequence of a primary sensitization to *Artemisia*.[16] Of course, in view of the limited number of serum samples tested, we cannot exclude that some Amb+/Art+, Art v 1+ patients with a 2-dimensional Amb a 1 isoform map similar to that of patients monosensitized to ragweed (and thus showing a true cosensitization to ragweed and mugwort) may exist. The existence of cosensitized patients might be one of the reasons for the discrepancy between our results and those reported by Asero and collaborators.[1] However, the lower protein concentrations of pollen extracts that Asero et al[1] used in their inhibition experiments compared with those we used in our experiments could also represent an additional explanation of the discrepancy between the 2 conclusions. In fact, given the high variability of serum IgE type and concentration, it is possible that, at least in some cases, the protein concentrations used in the previous work were not sufficient to induce a complete IgE-binding inhibition. In support of this hypothesis, most articles indicate the existence of significant cross-reactions between ragweed and mugwort allergens,[7,8,17,18] and in our opinion, at least in the Milan area, cross-reactivity is responsible for the double sensitization in most cases. Only an increase in the number of serum samples analyzed will allow a more complete understanding of these phenomena.

Furthermore, our findings allowed us to discriminate between Amb+/Art+, r Art v 1+ and Amb+/Art+, rArt v 1− patients; the latter also reacted against both Art v 6 and Amb a 1 (but not with Art v 1) and had a 2-dimensional Amb a 1 isoform map similar to that of patients monosensitized to ragweed (Amb+/Art−). In this case, our inhibition experiments revealed that the primary sensitizer was the ragweed major allergen Amb a 1. In agreement with a previous report that Amb a 1 is able to elicit a more diverse repertoire of IgE than Art v 6, specific for Amb a 1 in most cases but cross-reactive to Art v 6 in some others,[8] the serum samples from this third group of patients (Amb+/Art+, rArt v 1−) reacted to all Amb a 1 isoforms with a pattern that was indistinguishable from that of serum samples from Amb+/Art− patients. Thus, our experiments revealed that Art v 6 plays an important role in mugwort allergy, that the cross-reactivity between Art v 6 and Amb a 1 may be bidirectional, and that Art v 1 remains the only routinely available marker of a primary sensitization to mugwort. The inclusion of Art v 6 in commercial diagnostic allergen panels for in vitro diagnosis would be useful in view of the existence of Amb−/Art+, Art v 1− patients who react against Art v 6–specific, non–cross-reactive epitopes.[8] Nevertheless, in Amb+/Art+ patients, the detection of IgE to Art v 1 seems a fairly good marker to detect the primary source of sensitization because most patients clinically allergic only to mugwort react to Art v 1.[18] Allergens other than Amb a 1 and Art

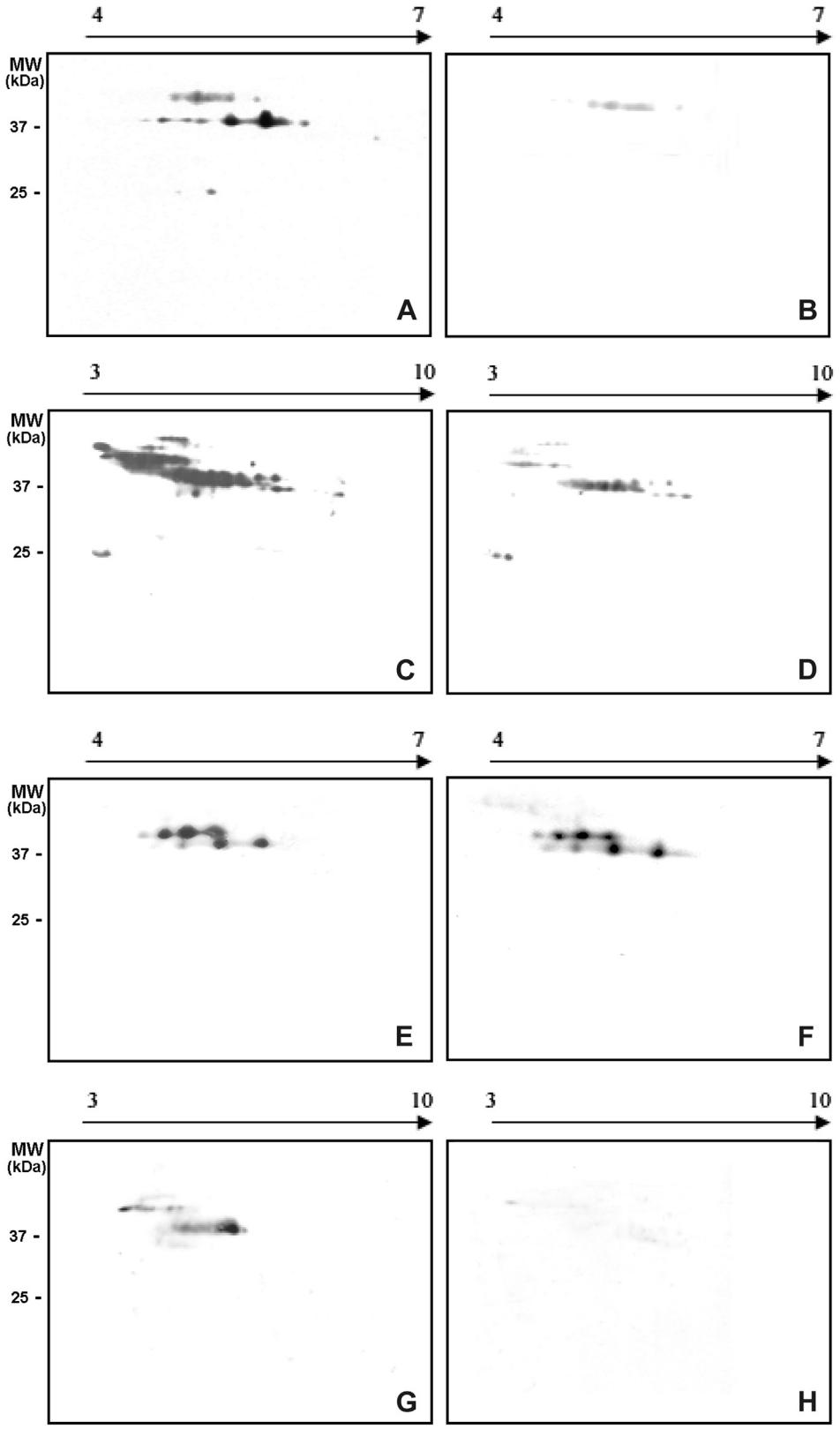

Figure 3. Two-dimensional immunoblot-inhibition assay. Representative pictures of reactivity of IgE contained in Amb+/Art+, recombinant Art (rArt) v 1+ patient serum samples to ragweed allergens detected before *(A)* and after *(B)* the absorption of sera with mugwort pollen extract. Representative pictures of reactivity of IgE contained in Amb+/Art+, rArt v 1+ patient serum samples to mugwort allergens detected before *(C)* and after *(D)* the absorption of sera with ragweed pollen extract. Representative pictures of reactivity of IgE contained in Amb+/Art+, rArt v 1− patient serum samples to ragweed allergens detected before *(A)* and after *(B)* the absorption of sera with mugwort pollen extract. Representative pictures of reactivity of IgE contained in Amb+/Art+, rArt v 1− patient serum samples to mugwort allergens detected before *(F)* and after *(G)* the absorption of sera with ragweed pollen extract. IgE reactivity against ragweed allergens of Art v 1+ serum is completely inhibited by preabsorption with whole mugwort extract, whereas preabsorption with whole ragweed extract has only a partial effect on IgE reactivity to mugwort allergens. In contrast, IgE reactivity against mugwort allergens of Art v 1− serum is completely inhibited by preabsorption with whole ragweed extract, whereas preabsorption with whole mugwort extracts exerts no effect on IgE reactivity to ragweed allergens.

v 6, such as Amb a 4, which is highly homologous to Art v 1,[7] might also play a role in sensitization to Compositae pollen and thus represent a further confounding factor. However, in our 2-dimensional immunoblotting, Amb a 4 was only a minor allergen and was never detected by serum samples from Amb+/Art+, Art v 1+ patients. A similar result was also obtained by Asero et al.[1] Thus, Art v 1 can be considered a good marker of *Artemisia* sensitization in Amb+/Art+ patients, although in the absence of a 2-dimensional immunomap of Amb a 1 isoforms, it is currently not possible to completely exclude a cosensitization.

Altogether, this study found that cross-reactivity between *Artemisia* and *Ambrosia* pollen exists, is frequent (at least in the area of Milan), is clinically relevant, and may be bidirectional. In clinical practice, the best marker of primary sensitization presently available is Art v 1, although it is not sufficient to discriminate between corecognition and cosensitization. In conclusion, by combining the results of SPT and proteomic analyses, we were able to determine the primary sensitizer in Amb+/Art+ patients, discriminating between cosensitization and corecognition. Unfortunately, this approach is currently not applicable in the clinical practice; using the currently available diagnostic tests, we found that Amb+/Art+ patients reactive to Art v 1 should be prescribed mugwort immunotherapy, whereas Amb+/Art+ patients not reactive to Art v 1 should be prescribed ragweed immunotherapy because ragweed is probably the primary sensitizer. Whether these patients should be also treated with ragweed or mugwort immunotherapy, respectively, remains an unanswered question in the routine clinical practice.

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References

[1] Asero R, Wopfner N, Gruber P, Gadermaier G, Ferreira F. *Artemisia* and *Ambrosia* hypersensitivity: co-sensitization or co-recognition? *Clin Exp Allergy*. 2006;36:658–665.

[2] Gallesio MT. Composite: Ambrosia. *Giorn It Allergol Immunol Clin*. 1991;1:467–474.

[3] Asero R, Venturini E, Minisini S, Coppolecchia AM, Massironi F, Velati C. Indagine sul contributo di diversi test in vivo e in vitro alla diagnosi differenziale tra pollinosi da *Artemisia* e da *Ambrosia*. *Giorn It Allergol Immunol Clin*. 1994;4:103–106.

[4] Mutschlechner S, Mari A, Van Ree R, et al. Patterns of Art v. 1 recognition in mugwort and ragweed-sensitized patients. Proceedings from the XXII Congress of the EAACI; 2003; Paris, France, 7-11 June 2003. Abstract 384.

[5] Park HS, Kim MJ, Moon HB. Antigenic relationship between mugwort and ragweed pollens by crossed immunoelectrophoresis. *J Korean Med Sci*. 1994;9:213–217.

[6] Weber RW. Patterns of pollen cross-allergenicity. *J Allergy Clin Immunol*. 2003;112:229–237.

[7] Leonard R, Wopfner N, Pabst M, et al. A new allergen from ragweed (*Ambrosia artemisiifolia*) with homology to Art v 1 from mugwort. *J Biol Chem*. 2010;285:27192–27200.

[8] Jahn-Schmid B, Hauser M, Wopfner N, et al. Humoral and cellular cross-reactivity between Amb a 1, the major ragweed pollen allergen, and its mugwort homolog Art v 6. *J Immunol*. 2012;188:1559–1567.

[9] Ferreira F, Hawranek T, Gruber P, Wopfner N, Mari A. Allergic cross-reactivity: from gene to the clinic. *Allergy*. 2004;59:243–267.

[10] Aina R, Asero R, Ghiani A, Marconi G, Albertini E, Citterio S. Exposure to cadmium-contaminated soils increases allergenicity of *Poa annua* L. pollen. *Allergy*. 2010;65:313–321.

[11] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248–254.

[12] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680–685.

[13] Han X, He L, Xin L, Shan B, Ma B, Peaks PTM. mass spectrometry based identification of peptides with unspecified modifications. *J Proteome Res*. 2011;10:2930–2936.

[14] Gadermaier G, Jahn-Schmid B, Vogel L, et al. Targeting the cysteine-stabilized fold of Art v 1 for immunotherapy of Artemisia pollen allergy. *Mol Immunol*. 2010;47:1292–1298.

[15] Jahn-Schmid B, Kelemen P, Himly M, et al. The T cell response to Art v 1, the major mugwort pollen allergen, is dominated by one epitope. *J Immunol*. 2002;169:6005–6011.

[16] Ackermann-Liebricha U, Schindlera C, Freib P, et al. Sensitisation to Ambrosia in Switzerland: a public health threat in waiting. *Swiss Med Wkly*. 2009;139:70–75.

[17] Hirschwehr R, Heppner C, Spitzauer S, et al. Identification of common allergenic structures in mugwort and ragweed pollen. *J Allergy Clin Immunol*. 1998;101:196–206.

[18] Gadermaier G, Wopfner N, Wallner M, et al. Array-based profiling of ragweed and mugwort pollen allergens. *Allergy*. 2008;63:1543–1549.