

Evaluation of the allergenic potential of Ginkgo biloba extracts

Roschanak Mossabeh, Dietrich Kraft, and Rudolf Valenta

Department of Pathophysiology, University of Vienna, Austria

Untersuchungen zur allergenen Aktivität von Ginkgo biloba-Extrakten

Zusammenfassung. Ginkgo biloba-Extrakte werden zur Behandlung von zerebralen und peripheren Durchblutungsstörungen, Hirnleistungsstörungen und des dementiellen Syndroms eingesetzt. Zwischen 1996 und 1998 wurde vermehrtes Auftreten von allergieähnlichen Symptomen bei parenteraler Applikation von Ginkgo biloba-Präparaten in Österreich berichtet. Ziel unserer Studie war es zu untersuchen, ob Ginkgo biloba-Extrakte Typ I-Allergene enthalten. Ginkgo biloba-Extrakte wurden auf Proteingehalt mittels Proteinbestimmung und Polyacrylamidgel-Elektrophorese untersucht. Wir verwendeten Seren von 95 polysensibilisierten Allergikern mit IgE-Antikörpern gegen die meisten pflanzlichen Allergene sowie polyklonale Hasenantisera, die gegen definierte rekombinante pflanzliche Allergene hergestellt wurden, um die Ginkgo-Präparate mittels dot-blotting und Westernblot auf das Vorhandensein von Allergenen zu untersuchen. Keines der ausgetesteten allergen-spezifischen Hasenantisera und Patientenserum reagierte mit den Ginkgo-Extrakten. Wir zeigen weiters, dass Ginkgo biloba-Extrakte auch durch einfache Hauttestuntersuchungen auf allergene Wirkung überprüft werden können. Zusammenfassend ergab sich kein Hinweis auf ein Vorhandensein von Typ I-Allergenen in Ginkgo biloba-Extrakten. Eine eventuelle Sensibilisierung kann vor der Applikation in jedem Fall, wie von uns beschrieben, durch eine serologische und/oder Hauttestuntersuchung abgeklärt werden.

Schlüsselwörter: Ginkgo biloba, Typ I-Allergie, IgE-Antikörper, Allergen.

Summary. Ginkgo biloba extracts are used for the treatment of central and peripheral malperfusion, cerebral insufficiency and dementia. Between 1996 and 1998, several patients in Austria who had received parenteral Ginkgo extracts were reported to have developed allergy-like symptoms. The aim of the present study was to determine whether Ginkgo biloba extracts contain type I allergens. The protein content of Ginkgo biloba extracts was determined by BCA protein determination and SDS-PAGE. We used sera from 95 polysensitized plant-allergic patients (the sera contained IgE antibodies against most plant allergens), and rabbit antisera raised against

defined recombinant plant allergens. The presence of allergens in Ginkgo extracts was determined by dot-blotting and Western blot. Neither rabbit antisera nor IgE antibodies of patients reacted to the Ginkgo extracts. In addition, it was shown that prick testing of the skin could be conveniently used to study Ginkgo extracts for allergenic activity. In conclusion, no evidence for the presence of type I allergens in Ginkgo extracts was found. We recommend serological and/or skin testing to exclude sensitisation to components of Ginkgo biloba extracts.

Key words: Ginkgo biloba, Type I allergy, IgE antibodies, allergen.

Introduction

Nearly 25% of the population suffer from type I allergy, an IgE-mediated hypersensitivity disease [1]. The symptoms of type I allergy (allergic rhinoconjunctivitis, asthma, dermatitis and anaphylactic shock) are due to IgE-mediated effects (i.e. IgE-induced effector cell and T-cell activation). Cross-linking of effector cell-bound IgE antibodies by allergens induces the release of biologically active mediators [2] and IgE-mediated presentation of allergens strongly induces T cell activation [3]. The disease-eliciting allergens mainly originate from airborne particles (e.g. pollen, mite faeces, mould spores, animal hair dander) and food [4] but may also be found in various plant extracts used for therapeutic applications (e.g. camomile) [5]. Plants contain a variety of structurally conserved proteins. The proteins exist as highly cross-reactive allergens in botanically unrelated plant species and different plant tissues. These include cytoskeletal proteins (e.g. profilin) [6, 7], calcium-binding proteins [8], and enzymes (polygalacturonases, pectinesterases) [9].

The aim of the study was to evaluate the allergenic potential of Ginkgo biloba preparations (Tebonin 17.5 mg, Tebonin 87.5 mg, Tebofortan 50 mg, Tebofortan 175 mg). These preparations are highly effective in the treatment of peripheral vascular occlusive disease, cerebral perfusion disturbances, cerebral insufficiency and Alzheimer's disease [10]. According to the pharmacological activity profile, Ginkgo biloba extracts are able to catch free radicals, antagonise the platelet-activating factor, exert positive effects on rheology and exhibit neuroprotective effects [10].

Between 1996 and 1998, several side effects similar to allergic reactions (e.g. urticaria, laryngeal oedema,

bradycardia, asthma, collapse, anaphylactoid reactions) were reported after parenteral application of Ginkgo biloba extracts (rates of side effects: Tebonin 17.5 mg: 0.0000054 %; Tebonin 87.5 mg: 0.0000236 %; Tebofortan 175 mg: 0.0000095%) in Austria. In about 77% of reported cases, the Ginkgo biloba preparations were applied by intravenous infusion along with different drugs as an infusion cocktail, although this form of administration is contraindicated and should have been avoided.

Ginkgo biloba extracts are prepared by standardised extraction of dried Ginkgo leaves [11]. They might contain ubiquitous plant allergens (e.g. profilin) and thus elicit type I allergic reactions in sensitised individuals. Since serum samples from patients who had experienced anaphylactoid side effects after administration of Ginkgo biloba extract were not available, we used serum samples from polysensitised allergic patients and rabbit antisera raised against frequent plant allergens, in order to determine the presence of allergens in Ginkgo biloba preparations. The allergen content of four parenteral Ginkgo biloba preparations (Tebonin 17.5 mg; Tebonin 87.5 mg; Tebofortan 50 mg; Tebofortan 175 mg) were examined by dot-blotting and Western blotting. Furthermore, the suitability of Ginkgo biloba preparations for prick testing of the skin was investigated. The anaphylactoid reactions observed thus far suggest that Ginkgo biloba extracts lack allergenic activity in this context. IgE serology and skin prick testing may be used as preventive measures to exclude type I allergic sensitisation to Ginkgo biloba prior to application.

Methods

Characterisation of patients' sera and of rabbit anti-allergen antisera

Sera from 95 polysensitised plant-allergic patients and 5 non-allergic individuals were used for the detection of allergens in Ginkgo biloba extracts. The diagnosis of type I plant allergy was based on case history indicative of type I allergy, prick testing of the skin and CAP RAST (Pharmacia, Uppsala, Sweden) serology as described [12].

Table 1 summarises total IgE levels and specific IgE levels to birch pollen, timothy grass pollen and mugwort pollen extract. Data relating to IgE reactivity to nitrocellulose-blotted and dot-blotted Ginkgo biloba extracts were included in the table. For certain sera, no quantitative CAP RAST data were available but the presence of pollen allergen-specific IgE antibodies was confirmed by IgE immunoblotting as described [12].

Protein determination, SDS-PAGE

The protein content of Ginkgo biloba extracts (Austroplant, Vienna, Austria) was determined by BCA assay (Pierce, Rockford, IL) as recommended by the manufacturer.

Lyophilised Ginkgo biloba extracts were analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis. Coomassie blue staining and Silver staining were performed to estimate the quality of proteins [13–15].

Immunoblotting, dot blotting

For Western blot analysis we separated 1 mg/10 cm per 12.5%-preparative gel of each of the 4 Ginkgo extracts (Tebonin 17.5 mg; Tebonin 87.5 mg; Tebofortan 50 mg; Tebofortan 175 mg) and transferred the proteins to nitrocellulose membranes

(Schleicher & Schuell, Dassel, Germany) by electroblotting [16]. For control purposes, birch pollen and timothy grass pollen extracts were also blotted onto nitrocellulose as described [12]. Sera from the 95 allergic individuals and, for control purposes, sera from 5 non-atopic individuals were used to determine nitrocellulose-blotted allergens as previously described [7].

In order to detect low molecular weight IgE-reactive components under native conditions, dot blot experiments were performed. Aliquots containing 6 µg protein of each Ginkgo biloba extract (Tebonin 17.5 mg; Tebonin 87.5 mg; Tebofortan 50 mg; Tebofortan 175 mg extracts) and, for control purposes of birch pollen extract, were dotted onto nitrocellulose and exposed to the same panel of sera as described for immunoblotting. Bound IgE antibodies were determined as previously described [7].

Nitrocellulose-blotted extracts were also exposed to rabbit antisera raised against purified recombinant allergens (rBet v 1, rPhl p 1, rPhl p 5, rPhl p 6, recombinant profilin from tobacco and birch) and the corresponding preimmune sera. Bound rabbit antibodies were detected with ¹²⁵I-labeled donkey anti-rabbit antibodies as previously described [7].

Prick testing of the skin

Prick tests were performed in two polysensitised plant allergic patients and one non-atopic individual. For this purpose 20-µl aliquots of the Ginkgo biloba extracts were placed on the patients' forearm at a distance of about 3 cm between individual application points, as recommended by Dreborg [17].

As controls, 0.9% NaCl negative control, histamine dihydrochloride-positive control, and a commercial birch- and timothy grass pollen extract (Allergopharma, Germany) were used. Immediate skin reactions were documented 20 minutes after testing by photographs and by transferring the wheal area encircled with a ballpoint pen to paper. Reactions sites were checked after 24 and 48 hours for potential late phase reactions.

Results

Ginkgo biloba preparations contain consistent amounts of low molecular weight proteins

Quantitative analysis of protein contents in different batches of Ginkgo biloba extracts revealed the presence of

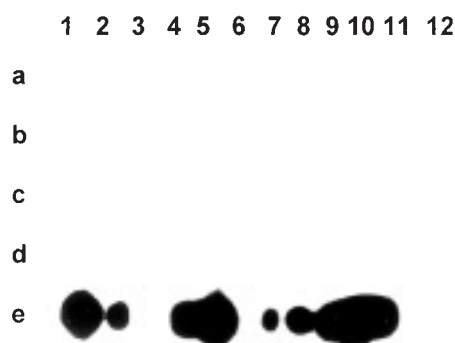


Fig. 1. IgE reactivity to dot-blotted extracts. Protein extracts from four Ginkgo preparations (a: Tebofortan 175 mg; b: Tebofortan 50 mg; c: Tebonin 87.5 mg; d: Tebonin 17.5 mg) and birch pollen extract (e) were dotted to nitrocellulose strips. Strips were exposed to sera from 11 pollen allergic patients (9 birch pollen allergic patients: lanes 1, 2, 4, 5, 7–11; two grass pollen allergic patients without birch pollen allergy lanes 3, 6) and a non-atopic person (lane 12)

Table 1. Serological characterisation of individuals

Individual	Total IgE(kU/l)	Specific IgE(kUA/l)			IgE to Ginkgo biloba extracts
		Birch	Timothy grass	Mugwort	
#1	>2000	>100	55.2	19.4	—
#2	1234	33.1	>100	7.92	—
#3	1587	20.0	>100	0.91	—
#4	1466	72.0	>100	3.02	—
#5	>2000	88.5	>100	46.7	—
#6	>2000	>100	49.3	2.40	—
#7	592	>100	4.10	0.55	—
#8	588	0.91	>100	<0.35	—
#9	560	>100	90.4	17.4	—
#10	460	>100	12.9	0.50	—
#11	543	33.3	>100	5.71	—
#12	1204	>100	36.0	4.86	—
#13	1642	77.0	n.d.	0.45	—
#14	403	65.6	4.73	3.33	—
#15	1733	>100	<0.35	<0.35	—
#16	320	57.7	<0.35	<0.35	—
#17	267	79.8	<0.35	0.49	—
#18	150	<0.35	33.6	<0.35	—
#19	141	<0.35	67.9	1.04	—
#20	>2000	n.d.	n.d.	n.d.	—
#21	>2000	>100	n.d.	15.2	—
#22	401	29.3	52.8	9.21	—
#23	>2000	>100	>100	16.0	—
#24	141	7.35	27.1	4.03	—
#25	124	7.50	<0.35	<0.35	—
#26	29.3	12.0	<0.35	<0.35	—
#27	238	9.74	7.83	16.0	—
#28	24.3	3.97	<0.35	<0.35	—
#29	225	<0.35	1.42	<0.35	—
#30	1274	67.8	>100	2.55	—
#31	93.5	4.14	17.0	<0.35	—
#32	184	2.41	78.1	1.74	—
#33	33.9	6.75	6.87	<0.35	—
#34	51.4	13.4	8.63	2.65	—
#35	371	6.17	45.1	3.41	—
#36	424	19.2	44.6	1.36	—
#37	204	22.1	37.7	1.13	—
#38	234	14.3	6.82	0.68	—
#39	1858	19.9	12.1	<0.35	—
#40	98.3	58.1	0.36	<0.35	—
#41	120	11.2	19.9	0.45	—
#42	34.1	14.4	<0.35	<0.35	—
#43	126	14.5	25.9	<0.35	—
#44	286	>100	<0.35	<0.35	—
#45	125	51.3	0.55	<0.35	—
#46	278	54.5	37.0	1.13	—
#47	230	<0.35	21.6	0.41	—
#48	111	10.2	13.4	<0.35	—
#49	364	0.98	39.5	1.30	—
#50	196	<0.35	5.2	<0.35	—
#51	401	31.3	89.5	<0.35	—
#52	n.d.	n.d.	n.d.	n.d.	—
#53	370	90.1	>100	1.31	—
#54	n.d.	n.d.	n.d.	n.d.	—
#55	40.6	<0.35	10.4	<0.35	—
#56	261	68.9	28.1	1.55	—
#57	129	<0.35	4.85	<0.35	—
#58	205	>100	<0.35	<0.35	—
#59	n.d.	n.d.	n.d.	n.d.	—

Table 1. (continued)

Individual	Total IgE(kU/l)	Specific IgE(kUA/l)			IgE to Ginkgo biloba extracts
		Birch	Timothy grass	Mugwort	
#60	158	20.5	5.0	<0.35	–
#61	326	0.38	1.57	0.85	–
#62	178	17.5	<0.35	<0.35	–
#63	172	34.6	1.76	1.97	–
#64	297	23.4	58.5	1.82	–
#65	124	41.7	<0.35	<0.35	–
#66	260	1.36	81.1	<0.35	–
#67	204	<0.35	1.39	<0.35	–
#68	315	38.1	>100	3.24	–
#69	732	24.8	2.12	2.21	–
#70	110	<0.35	3.71	0.37	–
#71	n.d.	n.d.	n.d.	n.d.	–
#72	207	30.2	83.9	3.26	–
#73	n.d.	n.d.	n.d.	n.d.	–
#74	153	30.8	1.38	<0.35	–
#75	n.d.	n.d.	n.d.	n.d.	–
#76	30.3	n.d.	n.d.	n.d.	–
#77	189	>100	<0.35	<0.35	–
#78	388	24.0	2.33	0.39	–
#79	354	69.7	>100	0.74	–
#80	209	5.84	44.6	2.39	–
#81	152	<0.35	29.9	<0.35	–
#82	206	62.5	5.04	2.41	–
#83	61.2	4.08	3.41	<0.35	–
#84	459	<0.35	1.59	1.24	–
#85	471	n.d.	n.d.	n.d.	–
#86	293	9.89	<0.35	<0.35	–
#87	189	15.3	14.4	0.56	–
#88	380	3.42	32.0	3.56	–
#89	260	87.5	1.18	<0.35	–
#90	406	<0.35	12.3	–	–
#91	309	32.0	7.68	6.45	–
#92	41.0	0.50	14.1	0.78	–
#93	152	29.0	1.02	0.62	–
#94	128	20.2	<0.35	<0.35	–
#95	162	28.2	11.8	<0.35	–
#96	n.d.	<0.35	<0.35	<0.35	–
#97	66.2	<0.35	<0.35	<0.35	–
#98	n.d.	<0.35	<0.35	<0.35	–
#99	9.50	<0.35	<0.35	<0.35	–
#100	n.d.	n.d.	n.d.	n.d.	–

kU/l kilo units per liter; kUA/l kilo units antigen per liter; n.d. not done; – no IgE reactivity.

consistent amounts of proteins in different extract batches. Tebofortan 175 mg/ml and Tebofortan 50 mg/ml typically contained 10 mg/ml and 6.2 mg/ml, respectively. Tebonin 87.5 mg and Tebonin 17.5 mg typically contained 0.6 mg/ml and 0.5 mg/ml, respectively. Qualitative analysis of the Ginkgo biloba proteins by SDS-PAGE and Coomassie Blue as well as Silver staining revealed the presence of proteins with a molecular weight of less than 40 kDa (data not shown).

Ginkgo biloba extracts lack IgE-reactive components

To look for IgE-reactive components in Ginkgo biloba extracts, we used sera from polysensitised plant-allergic patients (Table 1). Sera were selected to contain IgE antibodies against most plant allergen sources and allergens. Care was taken to include sera from polysensitised individuals with high levels of total serum IgE (>500 kU/L) as well as from patients with lower IgE levels but broad sensitisation to a variety of plant allergen sources. In ad-

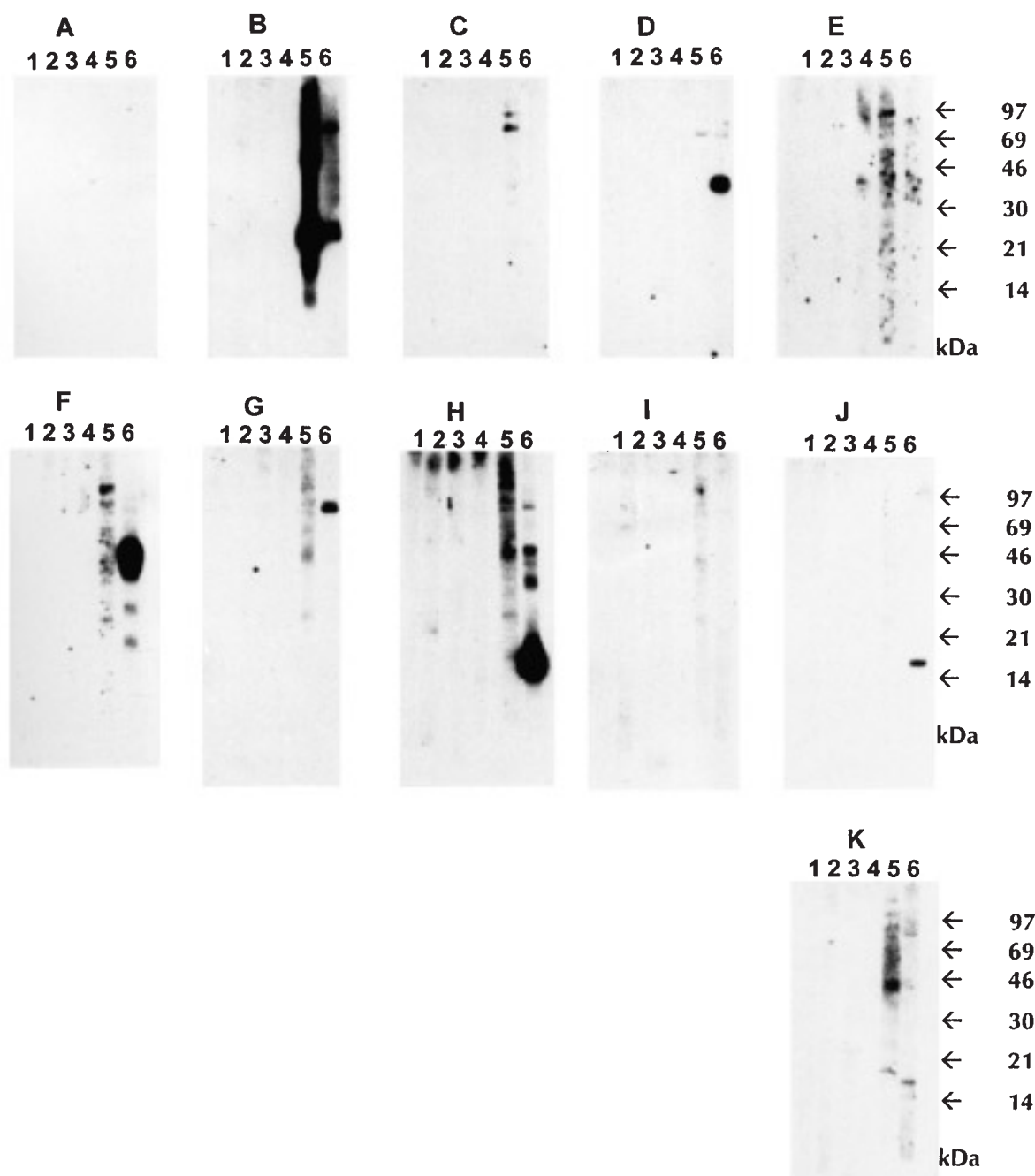


Fig. 2. Reactivity of allergen-specific rabbit antisera with nitrocellulose-blotted Ginkgo extracts. Nitrocellulose-blotted Ginkgo biloba extracts (lanes 1: Tebofortan 175 mg; lanes 2: Tebofortan 50 mg; lanes 3: Tebonin 87.5 mg; lanes 4: Tebonin 17.5 mg), birch pollen extract (lanes 5) and timothy grass pollen extract (lanes 6) were exposed to rabbit antisera. Panels B and A were exposed to rabbit-rBet v 1 antibodies and the corresponding preimmune serum, respectively. Panel D: rabbit anti-rPhl p 1 antibodies; panel C: preimmune serum; panel F: rabbit anti-rPhl p 5 antibodies; panel E: preimmune serum; panel H: rabbit anti-Phl p 6 antibodies; panel G: preimmune serum; panel J: rabbit anti-recombinant tobacco profilin; panel I: preimmune serum; panel K: rabbit anti-recombinant birch profilin

dition to IgE antibodies specific for pollen allergens, several patients had IgE antibodies against fruits, vegetables, spices and latex. Sera from the individuals in Table 1 were tested for IgE reactivity to nitrocellulose-blotted Ginkgo biloba extracts (Table 1), strong IgE reactivity to timothy grass pollen allergens was observed under identical experimental conditions (data not shown).

To prevent low molecular weight IgE-reactive components from being lost or destroyed during SDS-PAGE and Western blotting, dot blot experiments were carried out. As exemplified for 11 sera from allergic individuals and from a non-atopic person, no IgE reactivity was observed to dot-blotted Ginkgo extracts whereas birch pollen allergic patients reacted strongly to dotted birch pollen extracts (Fig. 1).

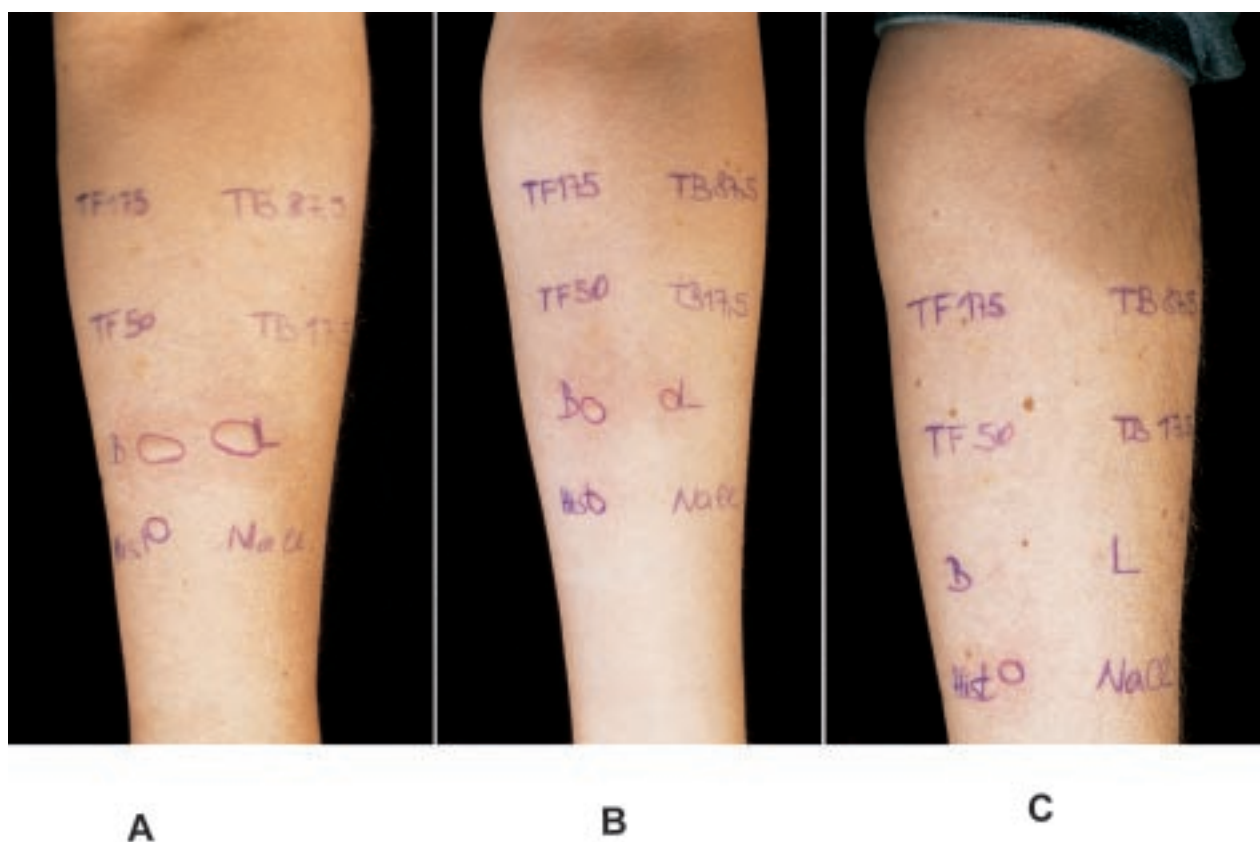


Fig. 3. Prick testing of the skin with Ginkgo biloba extracts. Two allergic (**A**, **B**) and one non-atopic individual (**C**) were pricked on their forearms with Ginkgo biloba extracts (*TF175*: Tebofortan 175 mg; *B*:87.5: Tebonin 87.5 mg; *TF50*: Tebofortan 50 mg; *TB17.5*: Tebonin 17.5 mg), birch pollen- (**B**) timothy grass pollen extract (*L*), histamine (*Hist*) and sodium chloride (*NaCl*). Wheal reactions were encircled with a ballpoint pen after 20 min

Absence of known plant allergens from Ginkgo biloba extracts

High-titer rabbit antisera which had been raised against several well defined recombinant plant allergens were investigated with respect to cross-reactive Ginkgo biloba allergens. Rabbit antisera raised against the major birch pollen allergen, Bet v 1, the timothy grass pollen allergens, Phl p 1, Phl p 5 and Phl p 6 [7, 12, 18, 19] and against the highly cross-reactive profilins from tobacco [20] and birch were exposed to nitrocellulose-blotted Ginkgo biloba extracts, birch pollen- and timothy grass pollen extracts (Fig. 2). While the rabbit antisera detected the corresponding pollen allergens (Bet v 1 at 17 kDa: Fig. 2B; Phl p 1 at 35 kDa: Fig. 2D; Phl p 5 at 35 kDa: Fig. 2E; Phl p 6 at 11 kDa: Figure 2H; profilins at 14 kDa: Figs. 2J, K) no reactivity with nitrocellulose-blotted Ginkgo extracts was observed (Fig. 2: lanes 1–4).

Ginkgo biloba extracts did not induce unspecific skin reactions: Prick testing of the skin can be used to study the potential allergenic activity of Ginkgo biloba extracts

Prick testing of the skin is a simple method to determine the allergenic activity of a given substance. Ginkgo biloba extracts were investigated in regard of whether they

could be used for prick testing of the skin in plant allergic patients and non-atopic controls without inducing toxic reactions. For this purpose we performed skin prick tests in two plant allergic patients (patient #54, and patient #83) and one non-atopic individual (patient #95) without Ginkgo biloba-specific IgE antibodies (Fig. 3). Birch pollen- and timothy grass pollen extract induced immediate skin reactions in sensitised individuals (Fig. 3A, B) but not in the non-atopic individual (Fig. 3C). Histamine (positive control) induced wheal and flare reactions, whereas sodium chloride (negative control) elicited reactions in all individuals (Fig. 3A–C). In agreement with serological data, Ginkgo biloba extracts did not induce skin reactions in any of the tested individuals (Fig. 3A–C).

Discussion

In the last few years, several patients in Austria have presented with allergy-like reactions (e. g., urticaria, laryngeal oedema, bradycardia, asthma, anaphylactoid reactions, collapse) shortly after treatment with parenteral Ginkgo biloba extracts. Ginkgo biloba extracts are prepared from dried Ginkgo leaves using a standardised extraction procedure which allows the production of extracts containing defined amounts of ginkgo flavonglycosides and terpenlactones, but no information as to the presence

of proteins with an allergenic potential is available [11]. The molecular characterisation of plant allergens by recombinant DNA technology has revealed the presence of cross-reactive allergens in various unrelated plant species (tree, grasses, weeds) and different plant tissues (pollen, fruits, vegetables) [21]. Cross-reactive plant allergens are proteins with important biological functions (e.g. cytoskeletal components, calcium-binding proteins, enzymes) and occur as structurally and immunologically related allergens in many allergen sources [22]. Therefore, patients who are sensitised to cross-reactive allergens may have allergic reactions upon contact with various allergenic sources. We used sera from plant-allergic patients and rabbit antisera raised against important plant allergens for the purpose of identifying allergens in Ginkgo biloba extracts. The results show that Ginkgo biloba extracts do not contain type I allergens. Thus, it is unlikely that the anaphylactoid reactions observed after parenteral application of Ginkgo biloba extracts were due to IgE antibody-mediated reactions. Other explanations for the observed reactions to Ginkgo biloba extracts are immune reactions that are mediated by antibodies other than IgE, by immune cells (e.g. antigen-presenting cells, T cells) or toxic reactions. The latter possibility should be considered because, in about 77 % of reported cases, the Ginkgo biloba preparations were administered by intravenous infusion along with several different drugs as an infusion cocktail, which is clearly contraindicated and should have been avoided.

We used IgE antibody measurements to nitrocellulose-blotted Ginkgo extracts, a method which can be used as a simple screening test to analyse sera from patients prior to application of Ginkgo extracts to exclude sensitisation. Moreover, we showed that prick testing of the skin with Ginkgo biloba extracts is a fast method that may be used even by the general practitioner to exclude type 1 allergic sensitisation to Ginkgo biloba. As shown in the present study, allergen-specific antibodies/antisera and marker sera from well characterised allergic patients may be used for screening plant extracts used for therapeutic application as well as for screening plant-derived food and other materials which are suspected to contain allergens. Such investigations will provide information about the allergenic potential of various new plant products introduced into the market.

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Correspondence: Prof. Rudolf Valenta, Department of Pathophysiology, AKH, University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria,
E-mail: Rudolf.Valenta@akh-wien.ac.at

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