

The Western blot is a highly sensitive and efficient technique in diagnosing allergy to wasp venom

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Summary

Background Diagnosis of allergy to wasp venom and decision to perform immunotherapy are based on the patient's history, along with skin and *in vitro* tests.

Objective Given the high prevalence of specific IgE also in non-allergic individuals, we evaluated the sensitivity and specificity of Western blots as a possible alternative to serum analyses of venom-specific IgE.

Methods Skin prick and/or intracutaneous tests were performed in 30 patients with allergy to wasp venom (generalized reaction following sting) along with serum analysis of venom-specific IgE (AlaSTAT microplate) and Western blots. Western blots were subsequently scanned and evaluated qualitatively and semiquantitatively by means of densitometry. Bands were scored 'positive' in cases of signal intensities beyond the mean plus 3 standard deviations of control sera. Twenty newborns (age 2–7 days) and 30 adults without systemic or increased local reactions to hymenoptera stings served as controls.

Results Western blot sensitivity reached 100% in the samples studied and was thus superior to the sensitivities of serum analysis of venom-specific IgE using AlaSTAT microplate assay (90%) and skin tests (87%). The sensitivity of detection of a phospholipase A1 and antigen 5-specific band was higher compared with a hyaluronidase-specific band (97%, 97% and 86%, respectively). Twenty-four out of twenty-nine (83%) patients exhibited specific IgE antibodies against at least three distinct allergens. With regard to the specificities, skin tests as well as AlaSTAT microplate assays were comparable (90% and 93%, respectively), whereas the specificity of the Western blots was 70% if the appearance of any single band was regarded as a positive result. However, when analysing the appearance of a specific band for antigen 5 or hyaluronidase the specificity and overall diagnostic value increased markedly, making it the most efficient test (specificity 97% and 100%, efficiency 96.8% and 93.2%, respectively).

Conclusion As allergy to wasp venom is a severe and potentially life threatening disease, false-negative test results need to be minimized. Therefore, the superiority of the Western blot with regard to sensitivity, specificity and overall efficiency makes this technique a valuable tool for its diagnosis.

Keywords: antigen 5, hyaluronidase, immunotherapy, *in vitro* diagnosis, negative predictive value, phospholipase, positive predictive value, sensitivity, specificity, Western blot

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Introduction

Allergy to hymenoptera venom is a classical IgE-mediated disease with a potentially fatal course. An effective means of treating this serious condition is available in the form of specific immunotherapy (SIT) [1,2] after the diagnosis has clearly been established based on the patients' history along with skin and *in vitro* tests. Given the severity and potentially life threatening course of this allergy, false-negative test results need to be minimized. Sensitivities of skin prick tests and serum analysis of venom-specific IgE reach 90% and 80%, respectively [3]. However, there is evidence that detection of wasp venom-specific IgE in the serum is error-prone with regard to false-negative results [4,5]. On the other hand, up to 25% of the general population have been shown to exhibit venom-specific IgE [6,7]. Therefore, several other test systems have been evaluated to increase the sensitivity and specificity in the diagnosis of hymenoptera venom allergy, such as sting challenge [8], flow cytometry [9], leukotriene release [10] and Western blot [11,12].

Allergen extracts consist of a complex mixture of proteins which can be separated by molecular size on SDS-PAGE. Three major allergens can be identified in wasp venom, namely phospholipase A1 (35 kDa; Ves v1), hyaluronidase (44 kDa; Ves v2) and antigen 5 (23 kDa; Ves v5) [13–15]. Following electrophoretic transfer of the separated proteins onto nitrocellulose membranes, incubation of these matrices with patient sera reveals the specific antibody pattern of an individual. Thus, more detailed information beyond the pure detection of venom-specific antibodies can be obtained utilizing this immunoblot strategy. Since this approach possibly facilitates the monitoring of SIT to hymenoptera venom [11,16,17], we were interested in evaluating its potential in establishing the diagnosis.

Methods

Patients

Sera from 30 consecutive adult patients (14 female, 16 male; age 16–71 years, mean: 48.2 ± 16.2 ; total IgE 207.8 ± 243.7 kU/L) with a characteristic history of systemic anaphylactic reactions to wasp stings were included in the study (Table 1). All of these patients presented at least with generalized urticaria and itching occurring within 30 min after a wasp sting. Additional symptoms of the patients are listed in Table 1 according to the classification of Mueller [3]. All patients identified the stinging insect as a wasp. Thirty adolescent or adult individuals (13 female, 17 male; age 16–75 years, mean: 39.2 ± 16.4 ; total IgE 132.8 ± 303.2) lacking symptoms

Table 1. Demographic and immunological data of patients with anaphylactic reactions to wasp stings

Patient No.	Sex	Age (Yr)	Müller class	Total IgE (kU/L)	Atopic status ¹	Skin test ²	Class	Blot ³
1	F	68	III	126	–	ST ⁺	3	A,H,P
2	F	35	II	446	RCAP	SPT [–] ICT [–]	3	A,P
3	F	18	II	57	–	ST ⁺	0	A,H,P
4	M	29	II	84	DA	SPT [–] ICT [–]	3	A,H,P
5	M	53	IV	148	–	ST ⁺	3	A,H,P,M
6	M	30	III	299	AD	ST ⁺	4	A,H,P
7	M	16	I	–	–	ST ⁺	2	A,H,P,M
8	M	28	II	668	RCAS	ST ⁺	4	H
9	M	64	III	108	–	SPT [–] ICT [–]	6	A,H,P
10	F	64	II	199	DA	ST ⁺	3	A,H,P,M
11	M	53	III	1252	RCAS	ST ⁺	3	A,H,P
12	F	56	III	12	–	ST ⁺	2	n.d.
13	F	64	II	159	RCAS	ST ⁺	2	A,H,P
14	M	54	II	131	RCAS	ST ⁺	3	A,H,P
15	F	59	III	46	–	ST ⁺	1	A,H,P
16	M	70	II	38	–	ST ⁺	2	A,H,P
17	F	71	III	282	AA	ST ⁺	1	A,H,P,M
18	M	54	II	34	RCAS	ST ⁺	0	A,H,P
19	M	16	III	300	AD	SPT [–] ICT ⁺	2	A,H,P
20	F	59	III	188	–	ST ⁺	0	A,H,P
21	F	41	I	109	–	ST ⁺	2	A,H,P
22	M	65	II	169	DA	ST ⁺	3	A,H,P,M
23	F	59	III	26	–	ST ⁺	2	A,H,P
24	F	43	III	51	–	ST ⁺	3	A,H,P
25	M	58	III	–	–	ST ⁺	3	A,H,P
26	M	47	I	165	–	ST ⁺	3	A,P
27	F	46	IV	189	RCAS	SPT [–] ICT [–]	2	A,H,P
28	M	43	III	65	DA	ST ⁺	3	A,P
29	M	50	II	151	–	ST ⁺	2	A,H,P
30	F	34	II	252	RCAS	ST ⁺	2	A,P

¹Atopic status. Individual history of rhinoconjunctivitis allergica saisonalis (RCAS) or perennialis (RCAP), atopic dermatitis (AD), drug allergy (DA) or allergic asthma bronchiale (AA), respectively. –: no individual history of atopic diseases.

²SPT: skin prick test; ICT: intracutaneous test; ST: SPT and ICT.

³Blot: immunoblots with specific bands for antigen 5 (A), hyaluronidase (H), phospholipase A1 (P) and minor antigen (M).

of allergy to hymenoptera venom (absence of a history of systemic anaphylactic reactions to hymenoptera stings and absence of increased local reactions, defined as an oedema exceeding 10 cm in diameter with a duration of more than 24 h [3]) and 20 newborns (age 2–7 days) who had never

been stung by hymenoptera served as controls. Patients and controls gave informed oral consent for the study under witness of a second physician.

Coded serum aliquots of all patients and controls were kept frozen at -20°C until IgE analysis and immunoblotting.

Skin tests

Skin prick tests (SPT; concentrations: 1–100 $\mu\text{g/mL}$) and/or intracutaneous tests (ICT; concentrations: 1 ng/mL–1 $\mu\text{g/mL}$) were performed using commercially available allergen extracts (ALK Scherax, Hamburg, Germany) in all individuals except for the newborns. Skin prick test responses were regarded as positive if the maximum weal diameter was ≥ 3 mm and the ratio of the allergen weal diameter to the histamine diameter (positive control) was at least 1.0 without reaction to negative control (saline). Intradermal tests were evaluated as positive if the maximum weal diameter was ≥ 5 mm. In all patients with a positive history of anaphylaxis after a wasp sting intracutaneous tests have been performed if SPT were negative. In adult controls, SPT and ICT have been performed.

Specific IgE

Specific IgE to wasp venom was performed utilizing the liquid-phase immunoassay AlaSTAT Microplate system (DPC Biermann, Bad Nauheim, Germany) according to the manufacturer's instructions.

Western blot analyses

Immunoblotting was performed with the AlaSTAT Ala-BLOT specific IgE test system (DPC, Bad Nauheim, Germany) according to the manufacturer's instructions. Briefly, wasp venom extract (obtained from Vespa Laboratories, Inc., Spring Mills, PA, USA) was electrophoresed by SDS-PAGE, electrotransferred to nitrocellulose membrane strips and then incubated with 50 μL of diluted patient sera for 120 min at room temperature (20 – 24°C). Anti-IgE monoclonal antibody labelled with the reporter enzyme alkaline phosphatase was immobilized at sites where patient IgE was bound to wasp venom extract on the membrane strip. By adding a chromogenic substrate for the reporter enzyme, wasp venom bands recognized by a patient's specific IgE were visualized. The colour development was stopped after 15 min. The bands obtained were quantified after scanning using the Biosoft Software (Biosoft, Oxford, UK). Signals in controls or patients were regarded as being positive if their intensity (i.e. relative area under the curve) was in excess of the intensity plus 3 standard deviations of the blots using negative control sera.

To identify individual bands, molecular masses were assessed by measuring the distance between the edge of the dye front and the centre of the band of interest. To calculate the molecular mass in kilodaltons (kDa), which allows identification of the respective allergen, a lot-specific formula provided by the manufacturer was used. To control for the specificity of the signals obtained, Western blot inhibition tests were performed. Therefore, 50 μL of diluted patient's serum was pre-incubated overnight with 200 μL of wasp venom extract in a concentration of 500 $\mu\text{g/mL}$ (wasp venom kindly provided by ALK Scherax, obtained from Vespa Laboratories Inc. and 250 μL buffer at 4°C).

Statistical analysis

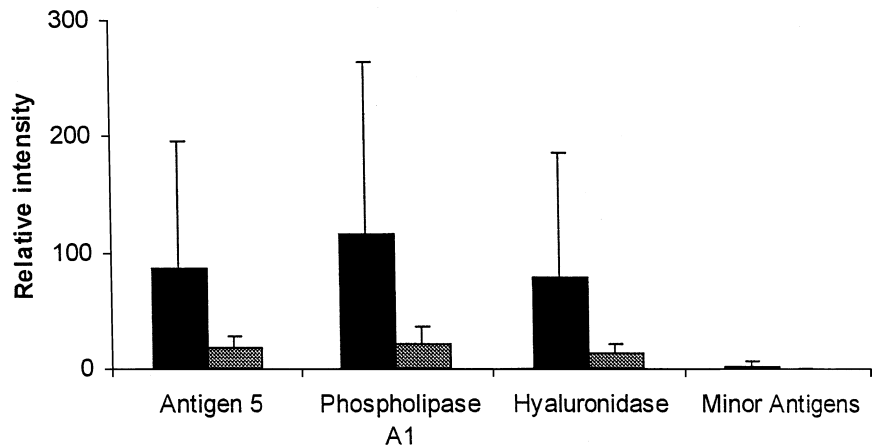
Data represent mean values $\pm$ standard deviation. Sensitivity was defined as the fraction of true positives among patients with a history of systemic anaphylactic reactions to wasp stings, specificity as the fraction of true negatives among controls without a history of systemic anaphylactic reactions or increased local reactions. Positive predictive value was defined as the likelihood of having the disease given that the test is positive, negative predictive value was defined as the likelihood of not having the disease given that the test is negative, and efficiency as the fraction of analysed individuals correctly classified by the test of interest [18].

Results

Sensitivity of skin and in vitro tests

First the sensitivity of skin tests utilizing commercially available wasp venom extracts was tested in a cohort of 30 individuals with a characteristic history of systemic anaphylactic reactions to wasp sting. Five out of 30 individuals with systemic anaphylactic reactions lacked positive results in this test, one of them turning positive on the ICT changing the sensitivity of the skin tests from 83% to 87%. In the AlaSTAT microplate analyses only three of the allergic individuals did not exhibit measurable specific IgE levels corresponding to a sensitivity of 90%. None of the patients with a negative wasp-specific IgE in the serum was also negative in SPT or in ICT. Twenty-nine out of 30 patients were analysed by immunoblotting. None of the patients with a history of anaphylactic reaction to wasp venom was missed by Western blot analysis. Twenty-eight out of 29 patients were positive for antigen 5 and phospholipase A1, whereas only 25/29 were positive for hyaluronidase, resulting in a sensitivity of 96.6%, 96.6% and 86.2%, respectively (Table 1 and Table 3). The molecular nature of the positive bands was identified by

Fig. 1. Western blot inhibition test reveals specificity of the major and minor allergen bands. Western blot and Western blot inhibition tests were performed as outlined in Materials and methods. Blots were scanned using Biosoft Software and the intensity of Western blots (black bars) compared with Western blot inhibition tests (hatched bars) is shown for each of the major allergens and for the minor antigen. The graph demonstrates an at least 80% inhibition of the band intensity when the patients' sera were pre-incubated overnight with wasp venom (500 µg/mL). Values represent means $\pm$ standard deviation.



calculating the size of the proteins according to the manufacturer's instruction. The specificity of the band was analysed by Western blot inhibition test. Western blot inhibition tests revealed a mean inhibition of at least 80% (Fig. 1).

Specificity of skin and in vitro tests

To test for the specificities of the respective diagnostic procedures we also analysed 30 adult individuals who had never experienced systemic anaphylactic or increased local reactions to hymenoptera venom, as well as 20 newborns aged 2–7 days who had never been stung by hymenoptera. None of the 30 adult non-allergic individuals exhibited positive skin prick test reactions to 1–100 µg/mL wasp venom extract, corresponding to a specificity of 100%. This number dropped to 90% when ICTs were performed. These individuals were only positive when tested with the highest concentration of 1 µg/mL. Serum IgE analyses revealed a sensitization towards wasp venom in two out of 30 controls (specificity: 93%), whereas wasp venom-specific IgE antibodies were not detected in any of the newborns (specificity 100%). In one out of two controls who showed a sensitization in the AlaSTAT microplate assay, ICT was also positive. In our study, the specificity of the wasp venom-specific IgE using AlaSTAT microplate assay is quite high compared with other studies that observed a sensitization of up to 25% in the general population [19]. Western blot analyses were positive in nine out of 30 adults and five out of 20 of the newborns, yielding specificities of 70% and 75%, respectively (Table 2).

Characterization of allergen spectra and specificity of Western blot

Having found positive signals in Western blots of all allergic individuals tested we wanted to further characterize

their respective IgE antibody patterns. IgE antibodies to all three major wasp venom allergens were detected in 24/29 sera tested. In five of those patients' sera also a 80–100 kDa minor antigen was detected. Four out of 29 patients were positive for antigen 5 and phospholipase A1 only and one out of 29 for hyaluronidase (Table 1). The signal intensities showed considerable variability, whereas none of the major allergens dominated the sensitization patterns obtained either inter-individually or intra-individually (Figs 1 and 2). The specificities of the respective signals were analysed by Western blot inhibition assays pre-absorbing specific IgE by adding wasp venom extract to the patients' sera prior to employing those sera in the conventional Western blots. Western blot inhibition showed a mean decrease of the signal intensity by at least 80% for each of the three major allergens. The signal of minor allergens could be blocked completely (Fig. 1).

In the adult control group, eight out of 30 adults had slightly positive bands for phospholipase A1, whereas one out of 30 was positive for antigen 5 and none for hyaluronidase (Table 2). This indicates a specificity for the individual phospholipase A1 band of 73%, of 97% for the antigen 5 band and of 100% for hyaluronidase in this limited number of controls (Tables 2 and 3).

Diagnostic performance of in vivo and in vitro tests

An overview of the diagnostic performance (sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), efficiency) of the different skin and *in vitro* tests is given in Table 3. PPV and NPV of the individual tests were calculated for a low and a high prevalence of hymenoptera allergy [20]. From the population investigated in this study we can conclude that detection of hyaluronidase by Western blot is the test with the highest PPV, whereas detection of antigen 5 by Western blot has the highest efficiency of 96.8%.

Table 2. Demographic and immunological data of adolescent or adult controls without a history of increased local reaction or anaphylactic reaction to hymenoptera stings

No.	Sex	Age (Yr)	Total IgE (kU/L)	Atopic status ¹	Skin test ²	Class	Blot ³
1	F	58	631	RCAS	ST ⁻	2	A
2	F	16	4	–	ST ⁻	0	–
3	M	48	11	–	ST ⁻	0	–
4	M	45	137	–	ST ⁻	0	–
5	M	55	18	–	ST ⁻	0	–
6	F	54	60	RCAS	ST ⁻	0	–
7	F	27	89	–	ST ⁻	0	–
8	M	23	20	–	ST ⁻	0	–
9	M	23	173	AD	SPT ⁺ ICT ⁺	0	–
10	M	30	61	–	ST ⁻	0	–
11	F	35	70	–	ST ⁻	0	–
12	M	25	145	RCAS	SPT ⁻ ICT ⁺	0	–
13	M	27	34	–	ST ⁻	0	–
14	M	45	16	–	ST ⁻	0	–
15	M	30	7	–	ST ⁻	0	–
16	M	35	17	–	ST ⁻	0	–
17	M	38	47	–	ST ⁻	0	–
18	M	70	11	–	ST ⁻	0	–
19	M	32	69	–	ST ⁻	0	–
20	F	32	4	–	ST ⁻	0	P
21	F	75	32	–	ST ⁻	0	–
22	F	28	5	–	ST ⁻	0	P
23	F	29	555	RCAS	SPT ⁻ ICT ⁺	2	P
24	M	30	43	–	ST ⁻	0	P
25	F	30	4	–	ST ⁻	0	–
26	F	27	142	RCAS	ST ⁻	0	P
27	F	72	17	–	ST ⁻	0	P
28	M	54	1538	AD	ST ⁻	0	–
29	M	37	10	–	ST ⁻	0	P
30	F	57	15	–	ST ⁻	0	P

¹Atopic status. Individual history of rhinoconjunctivitis allergica saisonalis (RCAS) or atopic dermatitis (AD), respectively. –: no individual history of atopic diseases.

²SPT: skin prick test; ICT: intracutaneous test; ST: SPT and ICT.

³Blot: immunoblots with specific bands for antigen 5 (A) or phospholipase A1 (P).

Discussion

This study documents in this cohort of patients and controls the superior sensitivity, specificity and efficiency of a Western blot technique for diagnosis of allergy to wasp venom when compared with standard diagnostic tests. Since this allergy represents a severe and potentially life threatening disease the first priority in the development of a

diagnostic procedure should be avoidance of false-negative results. Optimization of the test sensitivity by using Western blot techniques therefore represents valuable progress in establishing the diagnosis of wasp venom allergy.

This advantage, however, is associated with a loss of specificity if detection of at least one single wasp venom specific band is regarded as positive. From a practical point of view this disadvantage may be regarded as acceptable since the trade-off is not to miss any allergic individual. A possible explanation for this loss of specificity might be the influences the respective allergens are exposed to throughout the procedure of electrophoretic separation. Binding of IgE is dependent on the three-dimensional structure of the respective B cell epitopes. Therefore, the denaturing conditions under which SDS-PAGE is run [21] may interfere with the recognition of these epitopes. The consequence of this could be either failure of the IgE antibodies to recognize their respective epitope or loss of specificity of interaction. The former would result in loss of sensitivity, the latter could explain the overall low specificity of the Western blot. Another explanation for the low specificity of the immunoblot is the high prevalence of sensitization against bee or wasp venom [7], indicating that the immunoblot might not be false positive but detecting instead a state of low-grade sensitization that is – at least at the time of the evaluation – not of clinical importance.

Specificity of the Western blot signals can directly be tested by performing Western blot inhibition assays. In these assays we noted a reduction of signal intensity by more than 80% for all three major allergens of wasp venom; the much weaker signals of the minor allergens could be blocked completely. The markedly reduced intensity of the specific bands in blot inhibition assays indicates that the positive bands in control sera are probably not due to false positive results but mirror a high percentage of low-grade sensitization in our adult control group. Since IgE is (i) not transferred to the baby via the placenta, (ii) not (or only in very low concentrations) present in breast milk [22], and (iii) will be denatured in the stomach, detection of IgE binding to wasp venom in newborns who never experienced contact with hymenoptera venom is not to be expected.

Finally, persistence of a weak signal also in the Western blot inhibition assays may be due to different antigen sources or antigen compositions employed in the protein blots vs. pre-absorbance of specific IgE in the patients' sera. As DPC and ALK Scherax both obtain their venoms from the same company (Vespa Laboratories Inc.) different batches, different compositions of the antigen, or changes during the blotting procedure, may account for this phenomenon. The assumption that relatively small differences

Table 3. Evaluation of sensitivity, specificity and the predictive capacity of different clinically relevant diagnostic procedures in wasp venom allergy

Test	Sensitivity (%)	Specificity (%)	PPV ¹ (%)		NPV ² (%)		Efficiency
Prevalence ³			0.4%	3.3%	0.4%	3.3%	
Skin tests	87	90	3.4	22.9	99.9	99.5	88.5
Serum IgE	90	93	4.9	30.5	99.9	99.6	91.5
Western blot ⁴ (≥ 1 allergen)	100	70	1.3	10.2	100	100	85.0
Western blot ⁴ (Phospholipase A1)	97	73	1.4	10.9	100	100	84.6
Western blot ⁴ (Antigen 5)	97	97	11.5	52.4	100	99.9	96.8
Western blot ⁴ (Hyaluronidase)	86	100	100	100	99.9	99.5	93.2

¹PPV: positive predictive value.²NPV: negative predictive value.³Prevalence: PPV and NPV have been calculated for two different prevalences that have been published [20].⁴Western blot: sensitivity, specificity, PPV, NPV and efficiency of the Western blot were calculated for the occurrence of at least one positive band representative for the major allergens phospholipase A1, antigen 5 or hyaluronidase (≥ 1 allergen) or for the specific occurrence of one distinct band (either phospholipase A1 or antigen 5 or hyaluronidase).

between antigens caused by the respective sources may have an impact on the specificity of IgE absorbed is supported by the notion of small but measurable differences in skin prick test reactivity to native vs. recombinant phospholipase A₂ in individuals allergic to bee venom [23].

A loss of specificity in the immunoblot (70%) compared with skin tests (87%) and AlaSTAT microplate assay (93%) is only observable if the occurrence of any specific band in the Western blot is regarded as a positive result. Positive bands specific in healthy controls most often occur for phospholipase A1 (8/30), whereas a positive band for antigen 5 is found in only one and for hyaluronidase in none of the 30 controls. Therefore, when we focus on the detection of a specific band for antigen 5 or hyaluronidase the values for the specificity of the immunoblot change markedly to 97% and 100%, respectively. Therefore, we suggest focusing on antigen 5 and hyaluronidase-specific bands when analysing wasp venom immunoblots. The reason why the specificity of antigen 5 and hyaluronidase immunoblot is markedly higher compared with phospholipase A1 immunoblot remains unclear. One possible explanation might be the high degree of sequence identity between hymenoptera-derived phospholipase A1 (such as Dol m 1) with mammalian lipases [24].

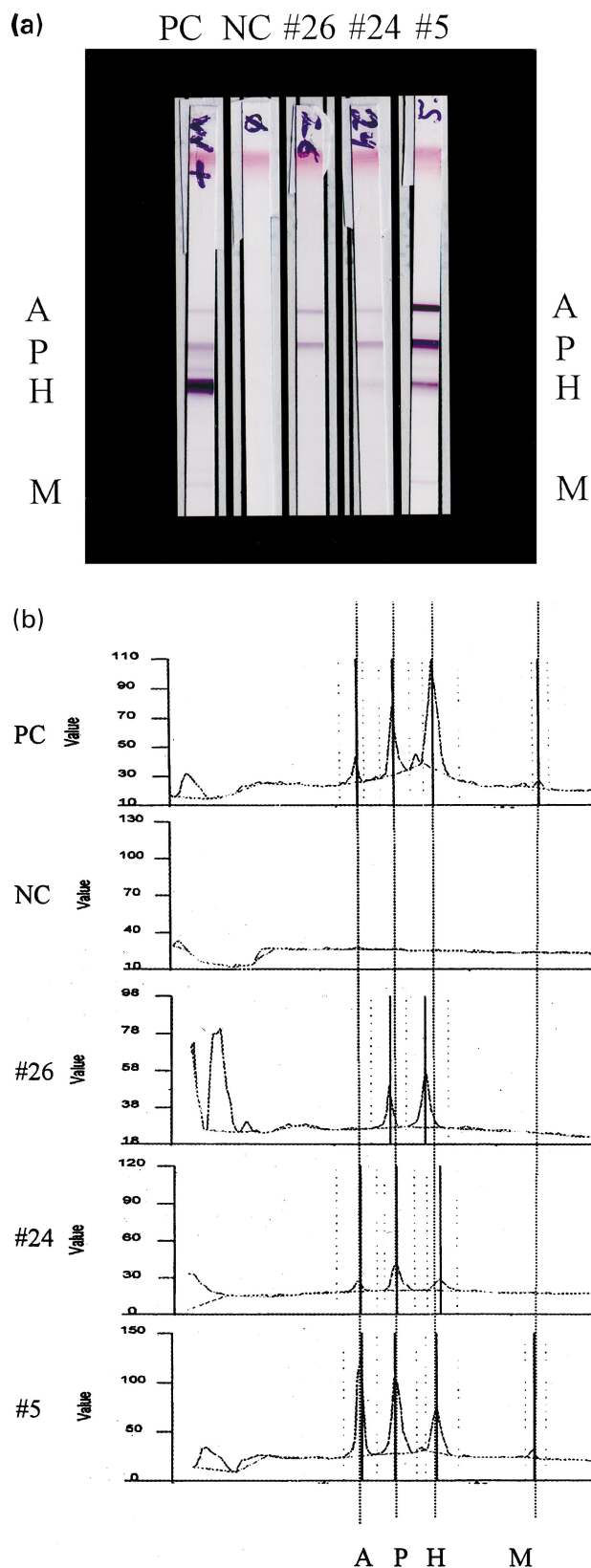
Allergy to hymenoptera venom can effectively be treated by means of specific immunotherapy [1,25]. Currently, venom extracts are used as vaccines, but attempts are under way to use recombinant technology to produce vaccines

that can be standardized to an even higher degree [26]. Additionally, many groups are involved in defining major allergens within complex extracts and even immunodominant epitopes, which ultimately should allow the production of patient-tailored vaccines that also minimize side-effects [27]. In this regard the observation is of relevance that specific IgE antibodies to each of the three major allergens in wasp venom can be detected in the sera from 24/29 allergic individuals included in this study. Consequently, an effective vaccine for these patients must contain all three proteins and the concept of individual vaccines does not seem to be feasible in the case of allergy to wasp venom, at least in the population studied in this report.

In conclusion, the superior sensitivity and specificity of Western blot analysis, especially when considering specific bands for antigen 5 and hyaluronidase, compared with standard diagnostic procedures makes this technique a valuable tool in establishing the diagnosis of allergy to wasp venom. The broad spectrum of sensitization documented by this technique also suggests that the development of an individualized specific immunotherapy for patients allergic to wasp venom seems not to be feasible. However, further studies are needed for confirmation of these initial results.

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Fig. 2. Binding of IgE to wasp venom components in immunoblots. Original immunoblots from three different patients representative for the three different reaction patterns are depicted. (a) Lane 1: Positive control (PC); lane 2: negative control (NC); lane 3: serum from patient #26 showing bands for antigen 5 and phospholipase A1 (#26); serum from patient #24 with positive staining for antigen 5, phospholipase A1, and hyaluronidase (#24); serum from patient #5 with positive staining for the above mentioned major allergens and minor antigen (#5). The relative intensity and relative position from the starting point (left) is represented in horizontal scans. (b) Again, PC, NC and sera from patients #26, #24 and #5 are shown. Vertical dotted lines indicate the positions of antigen 5 (A), phospholipase A1 (P), hyaluronidase (H) and minor antigen (M); horizontal dotted lines represent the relative intensity of the individual strip as background.

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